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THE UNIVERSITY OF ALBERTA

PHYTOPATHOGEN-INDUCED EXPRESSION OF A *PAL1* PROMOTER-LUCIFERASE GENE FUSION IN *ARABIDOPSIS THALIANA*

BY

LAWRENCE GIACOMIN ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

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The undersigned certify that they have read and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Phytopathogen-induced expression of a *PAL1* promoter-luciferase gene fusion in *Arabidopsis thaliana*, submitted by Lawrence Giacomin in partial fulfilment of the requirement for the degree of Masters of Science.

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ABSTRACT

Efforts to study the regulation of a phenylalanine-ammonia-lyase gene in *Arabidopsis thaliana*, in response to infection by phytopathogenic bacteria, focused on creating a novel *in planta* monitoring system. The *lux F* reporter gene, derived from *Vibrio harveyi*, was fused to the *Arabidopsis PAL1* promoter and this *PAL1-lux F* transcriptional fusion construct was introduced into *Arabidopsis* plants.

The *PAL1* promoter was amplified from genomic DNA using PCR and specific *PAL1* oligonucleotide primers determined from published data. The 1.8 kbp *PAL1* promoter was sequenced to confirm its identity. This promoter was then cloned into a plant expression vector, transcriptionally-fused to the *lux F* reporter gene, to form pGL101. *Arabidopsis* plants were transformed with pGL101 using *Agrobacterium*-mediated transformation techniques. To document the presence of the transgene, plant genomic DNA from the selfed-progeny of individual transformants was examined by PCR, using both specific *PAL1* and *lux A* oligonucleotide primers. The nontransformed plants possessed only the resident *PAL1* promoter sequences, while transformants possessed both the *PAL1* promoter and the *lux A* subdomain of the *lux F* reporter gene. Genomic DNA was further characterized by Southern hybridization demonstrating that the transformants were in fact unique transformants since the number and the nature of the T-DNA insertions were different in the individual plant lines analyzed.

Avirulent and virulent *Pseudomonas syringae* pathovars were infiltrated into *Arabidopsis* leaves to determine whether the *lux F* reporter gene was under the regulated control of the *PAL1* promoter. Enzymatic luciferase assays performed *in vitro* on

infiltrated leaves demonstrated that the avirulent bacteria caused a 3-4-fold increase in the luciferase activity, 24-72 hrs after infiltration. In contrast, leaves infiltrated with the virulent bacteria yielded only minimal increases in luciferase activity. Therefore, the *PAL1* promoter is specifically induced by the avirulent pathogen.

One transgenic line exhibited high luciferase activity and contained three T-DNA insertions. Hence a correlation between the copy-number of transgenes and the corresponding reporter enzyme activity was also demonstrated. Furthermore, these high levels of *PAL1-lux F* expression, induced after phytopathogen infection, allowed monitoring *in planta* using a computer-aided, single-photon-counting, camera system.

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LIST OF ABBREVIATIONS

dATP	deoxyadenosinetriphosphate
bp(s)	base pair(s)
BSA	bovine serum albumin
Ci	Curies
CTAB	hexadecyltrimethylammonium bromide
(d)dNTP's	(di-)deoxynucleotide triphosohates
EGTA	ethylene-bis(oxyethylennitrilo)tetraacetic acid
EDTA	ethylene-diamine tetraacetic acid
EtBr	ethidium bromide
g	force of gravity
hr(s)	hour(s)
IPTG	isopropyl-β-D thiogalactoside
kDa	kilodalton
LB	Luria-Bertani medium
min(s)	minute(s)
OAc	acetate ion (CH_3COO^-)
PAL	Phenylalanine-ammonia-lyase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	standard saline-citrate
T-DNA	transfer DNA
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TBST	tris-buffered saline with Tween 20
TE	tris-EDTA-buffer
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
YEB	yeast extract broth medium
UV	ultraviolet (light)
X-gal	5-bromo-4chloro-3indoyl-galactoside

I. INTRODUCTION

I.1 GENERAL PERSPECTIVES AND CHALLENGES

In nature plants exist in an exposed environment populated with many and diverse species of organisms. On average 100 square meters of temperate zone meadow at the height of summer will contain 150 000 individual plants. Associated with these plants in this same 100 square meters will be algae, protozoa, fungi and 1 000 billion bacteria, both above ground in the phyllosphere and below ground in the rhizosphere (Swain, 1977). Plants remain completely exposed to environmental microbes. As a result, plant surfaces become habitats for numerous species of bacteria and fungi. In fact, both the aerial, as well as the subterranean parts of plants serve as natural habitats for a variety of microorganisms, whose populations are strongly influenced by the overall, everchanging, environmental conditions (Beringer and Johnston, 1984).

The primary reason for a microbe to associate with the photoauxotrophic plant is to draw upon the plant's enormous reserves of fixed carbon and thereby satisfy its own nutritional requirements for carbon and energy. Plants are the primary producers of fixed carbon in the biogeochemical cycling of carbon and so provide an energy and a fixed carbon source for a wide range of heterotrophic organisms living on the earth (Atlas, 1984).

When in contact with a living plant microorganisms may live in a passive association feeding on plant exudate, microorganisms may coexist with the plant in a symbiotic relationship, or microbes may infect a plant in a pathogenic relationship which can cause damage to the plant and possibly result in plant disease. Disease is a major factor limiting crop productivity (Doerner *et al.*, 1990). In broad terms it has been estimated that about 30% of the total world crop production is lost every year and about one-third of this loss is due to pant diseases. On a yearly basis, the total global monetary losses in agriculture, due to plant pathogens, was estimated to be $49.6 billion U.S. (Goto, 1992). The precise quantitative assessment of crop losses due to diseases is a complex subject and this statistic remains an estimation. However, in specific terms there have been notable cases in the past where crop diseases caused by specific microbial pathogens have spread, leading to pandemics, culminating in complete crop failures. The potato famine of Ireland in the mid 1880's, caused by *Phytophthora infestans* and more recently, the corn leaf-blight epidemic of 1970-71 in the corn belt of the United States, caused by *Helminthosporium maydis* (Race T), are two prominent examples of acute pandemics causing tremendous losses in agriculture (Bills, 1977; Ullstrup, 1978).

Increasing world population trends has lead to the calculation that overall world crop productivity must double in the next 25-50 years to meet the expanding world population need's of food and fibre (Bongaarts, 1994; Bilger, 1992). In addition, the application of agrichemicals for plant protection can represent a substantial cost in bringing a crop to harvest and an important goal of breeding programs has been to reduce losses by exploiting the genetic variation of disease resistance within accessible gene pools (Doerner *et al.* 1990). Concerns about the health risks and environmental pollution have resulted in the widespread insistence for the application of less toxic agrichemicals for plant disease control. In order for agriculture to surmount these challenges, improved varieties of crops possessing resistance to primary pathogens must be developed at a faster pace. With the advances in molecular genetic techniques and plant gene-transfer technologies, the goal of genetically engineering microbial resistance in plants by increasing the effectiveness of the plant's own surveillance mechanism and defense responses, is becoming feasible. Nonetheless, for such a significant step to occur within agriculture, the basic communication between plants and microbes must first be examined and understood. In recent years scientists have begun to decipher, in a rudimentary way, the complex language and interaction between plants and microbial pathogens.

I.2 OVERVIEW OF PLANT-MICROBE INTERACTIONS

When a microbe comes into contact with a plant, the microbe may form a passive relationship, it may coexist with the plant symbiotically, or it may invade a plant in a pathogenic relationship. Only a few specific species of the tens of thousands of microbes encountered by a plant in nature will be capable of ever establishing a symbiotic or pathogenic relationship. By far the most common plant-microbe interaction can be described as a "null" interaction or a nonpathogenic reaction which is typified by microbes living in "harmony" with healthy growing plants; plants flourish worldwide and plant diseases are the exception rather than the rule (Lamb *et al.*, 1989). When a plant encounters a potential pathogen, the plant may respond by inducing a variety of defense genes and by reacting in a "hypersensitive" manner to contain the invasion of the pathogen, or it may respond by simply succumbing to the invader resulting in pathogenesis and plant disease. Complicated defense mechanisms do not guarantee resistance since the pathogen may produce virulence factors that compromise, or destroy components of the plants defence. If the pathogen surmounts the static defense mechanisms and does not display factors that elicit active plant defense reactions, disease may occur. Whereas the pathogen that fails to evade one or more components of the plant defense, generally will be unsuccessful (Keen, 1992). Hence, the plant posseses both passive and active resistance mechanisms. It is apparent that in active resistance there exists a two way communication between plant and microbe and results of the recent years have deciphered some of the features of this communication (Lamb *et al.*, 1989; Lamb, 1994).

I.3 PLANT RESITANCE MECHANISMS

I.3.1 Passive Resistance

Plants posses a variety of resistance mechanisms which can be broadly classified into two categories: i) passive and ii) active (Crute *et al.*, 1985). They can be distinguished by the fact that a passive, or preformed resistance mechanism, constitutes a normal part of a healthy uninfected plant's physiological "make-up" and requires no further energy input once it is established. In contrast, an active mechanism requires additional energy and is only brought into operation as a result of microbial challenge to a plant. Some passive resistance properties include: the hardness and the thickness of the cuticle and other surface tissue waxes, the wettability of surface tissue, morphological features such as the number and the structure of stomata and other natural pores, the dimensions of xylem vessels and other vascular tissues and constitutively produced antimicrobial chemicals and inhibitors of microbial produced plant cell wall degrading enzymes; all the formentioned features, plus many more, provide plants with a general passive resistance (Crute *et al.*, 1985).

For an organism to be parasitic in a particular host species it must have the capacity to breach and penetrate tissue, to be insensitive to passive chemical antimicrobial compounds and in general must be able to render ineffective the whole array of passive plant resistance mechanisms. Since most microbial parasites are restricted in their host-range it is believed that passive mechanisms have a considerable role in "non-host" or nonspecific resistance. Nevertheless it must be concluded that the major differences in the susceptibility of closely related cultivars to potential pathogens can rarely be attributed to passive resistance mechanisms alone (Crute *et al.*, 1985). Active defense mechanisms, expressed *de novo* upon exposure to a microbial pathogen, appear to be of primary importance in cultivar-specific plant resistance to microbes. In addition morphological characteristics such as the architecture of vascular tissue, or the thickness of cutin, are likely to be under the control of numerous pleiotrophic genes; such physiological traits are not amenable to modification by current genetic engineering techniques. Accordingly, active plant defense mechanisms, which genetic analysis over the years has shown appears to be dependent upon single plant loci, are of significant interest to molecular biologists studying plant-pathogen interactions.

I.3.2 Active Resistance Mechanisms

Plants possess several defense mechanisms which can be induced shortly after ingress by a potential pathogen. The active nature of the plant defense response was first clearly documented in the 1970's when it was shown that mRNA and protein synthesis were often required for the expression of host-resistance and that the appearance of "molecular defensive barriers" required the *de novo* synthesis of specific enzymes. The extent and strength of the expression of these defence mechanisms can determine whether a pathogen is successful forming what is known as a "compatible" reaction or whether a pathogen is unsuccessful forming what is known as "incompatible" reaction (Lamb *et al.*, 1989; Dixon and Harrison, 1990); the mechanism of control and activation of plant defense responses are of fundamental importance. Particular active plant defense mechanisms include the production of: i) reactive oxygen intermedites (e.g., superoxide anion and hydrogen peroxide), ii) hydroxyproline-rich glycoproteins (HRGPs) used in strengthening neighbouring undamaged tissue, iii) hydrolytic enzymes putatively involved in degrading microbial cell walls (e.g., chitinases and β-1-3 glucanases), iv) lignin and suberin which strengthen the plant cell wall and v) antimicrobial compounds called phytoalexins (Bell, 1981; Collinge and Slusarenko, 1989; Lamb, 1994). Lignification and phytoalexin production are the two active plant defense mechanisms which are relevant to this thesis and will be discussed in more detail.

I.3.3 Lignification

Lignin is one of the most abundant biopolymers on earth and is formed in healthy plant tissue, although it is prevalent mostly in woody plants (Vance *et al.*, 1980). Lignin or "ligno-suberin" stabilizes the overall structure of the plant cell wall and is highly resistant to degradation by most microorganisms (Collinge and Slusarenko, 1989; Kolattukudy, 1980 and 1981). Increased lignin deposition, in and around a plant wound, or at the site of pathogen attack, is a well documented active plant defense mechanism. Lignification of plant cell walls is one of the most common responses of plant tissues to wounding and infection, yet both wounding and infection may be required to induce *de novo* synthesis of lignin (Aist, 1985). Lignification is the covalent binding and polymerization, principally of sinapyl, coniferyl and *p*-coumaryl alcohols, in plant cell walls or related matrices (Vance *et al.*, 1980). Other phenylpropanoids such as *p*-hydroxy benzoic, ferulic and *p*-coumaric acids can be esterified to the polymer. Interestingly disease-induced lignin and non-disease lignin have been analyzed for types of chemical building blocks and chemical bonds and have been found to differ at this level. The significance of this property is not fully known, however, it is hypothesized that the disease-induced lignin may be more resistant to microbial degradation (Vance *et al.*, 1980).

Lignin infiltrates the spaces between the cell wall microfibrils, increasing the mechanical strength and decreasing the permeability and degradiblity of the plant cell walls (Aist, 1985). Lignification has thus been associated with resistance in many plant host-parasite associations and is part of the demarcation process whereby the expansion of lesions seems to be checked. The occurrence, speed and extent of lignification has been related to resistance in many host-parasite interactions (Bell, 1981). Lignified areas which are formed in response to microbial attack are resistant to cell wall degrading enzymes. The resistance to enzymatic maceration provided by lignification was demonstrated by Ride *et al.* (1975). Wounded wheat leaves inoculated with a variety of microbes showed heavy levels of induced lignification, which subsequently conferred resistance to tissue maceration in the surrounding lesion. Mechanical wounding alone, or exposure to a series of abiotic elicitors, which could induce phytoalexin biosynthesis, did not induce lignification. Fungi were necessary for the highest levels lignification. When pathogens were infiltrated into the wounded tissue they spread into nonwounded tissue and lignification appeared later and was weaker than in the tissue inoculated with nonpathogenic fungi. The ability of the plant to quickly lay down a dense lignin barrier correlates with its resistance to fungal pathogens.

Some of the first evidence to focus on induced lignification as a possible mechanism of plant disease resistance was provided by Akai (1953) who discovered the induced lignification on underground parts of numerous dicots in response to infection by *Helicobasidium mompa.* Root cells at the margin of the rot became lignified and additional corky layers formed deeper in the tissue. The fungus was able to penetrate the first layer of lignified cells in sweet potato and mulberry, yet the additional corky layers which formed deeper in the cortex did check the penetration of the fungus.

Some early evidence of lignification as a possible mechanism of plant disease resistance was provided by Hijwegen (1963). He demonstrated that the cortical cells of scab-resistant cucumber hypocotyls, when inoculated by the *Cladosporium cucumerinum*, showed high levels of lignin or lignin-like compounds five days after inoculation. This reaction was not detected in infected hypocotyls from susceptible cucumber varieties, or in healthy uninoculated hypocotyls. A report of the deposition of lignin, or lignin-like materials, in the dead cells of resistant cucumber was also made previously (Vance *et al.*, 1980). In this report it was shown that cucumber cells also formed the same lignin compounds in tissue wounded by brushing, cutting, or scratching in the absence of a pathogen, and susceptible tissue that was pretreated in this way, before inoculation, became resistant to what had been a previously virulent fungus. Recent studies on cucurbit diseases caused by *C. cucumerinum* and *C. lagenarium* substantiate the concept that systemic protection of "immunized" plants is associated with rapid lignification of cells beneath and surrounding spores or appresoria of the fungi (Vance *et al.*, 1980).

Lignification is involved in resistance to "shot-hold" diseases of leaves from *Prunus* sp. caused by *Clasteosporium carpophlum* (Vance *et al.*, 1980). Plant mesophyll cells, at a distance from tissue colonized by this fungus, swelled and occluded the intercellular spaces and the swollen cell layers nearest the infection site contained lignin and/or lignin-like compounds. Cells at the outer part of the occluded region assumed meristematic characteristics. If the leaf was young and the moisture was adequate an abscission layer formed in the meristematic region and the infected tissue dropped away. The remaining leaf cells of the meristematic zone then became partly lignified and suberized. It was suggested that a major factor in the breakage of the abscission layer was the rigidity of the lignified area in the infected tissue.

Tthe rapid deposition of lignin in potato tuber tissue was observed as a response to inoculation with phytopathogenic fungi that were nonpathogenic on potato. Potato tuber tissue inoculated with a pathogen *Fusarium roseum* f. sp. *sambucinum* demonstrated little deposition of lignin, while the same tissues inoculated with *C. cucumerinum* and *F. roseum*, pathogens of other plant species, exhibited high levels of lignin deposition (Hammerschmidt, 1984). In a similiar report, increases in both PAL enzyme activity and lignin-like compounds were observed as race-specific resistance response in different varieties of potato tubers, to specific races of *P. infestans*

(Henderson and Friend, 1979). Only the specific races of fungi that were avirulent on the corresponding varieties of potato induced high PAL activities and lignification.

The protection of carrot cells against the fungus *Botrytis cinera* by "pretreatment" with *B. cinera* killed-spores was demonstrated (Heale and Sharman, 1977). When the "pretreated" callus cultures were inoculated with live spores, extensive lignin formation occurred within 12 hrs before the live spores germinated. In "uninoculated-pretreated" or in "uninoculated-untreated" tissue, only minor amounts of lignification were detected. When the same experiments were carried out with carrot root-slices, the roots showed a similar mechanism of defense, although instead of lignin, increased levels of suberin were detected.

Suberin is a biopolymer similar in structure to lignin and also occurs as a normal component of healthy plant tissue. It is particularly present in roots and tubers and forms an impermeable moisture barrier protecting plant tissues from drying out (Kolattukudy, 1980 and 1981). Infection and wounding can trigger suberization of plant cell walls and this response could help to prevent the spread of an invading microbe since suberized cells are rarely penetrated by microbes (Bell, 1981). Suberization has been less studied as an active plant resistance mechanism than lignification. However, these two polymers have similar structures and indubitably share many antimicrobial features (Kolattukudy, 1980 and 1981). Chemical analysis has lead to models for the molecular structure and deposition of suberin (Kolattukudy, 1980 and 1981). The deposition of suberin typically occurs in layers alternating with waxes, in between the cell wall and the plasma membrane, thus conferring a layered profile when viewed under the electron microscope. At the molecular level suberin deposition is thought to occur in three stages: i) the covalent attachment of phenylpropane derivatives to plant cell wall polysaccharides, ii) the polymerization of additional phenylpropanoids until a phenolic matrix is built up and

iii) the attachment of aliphatic compounds to the matrix by ester bonds. Suberization thus resembles lignification to a significant degree, the principal difference being the additional aliphatic component of suberin, which usually consists of long chain fatty acids (C_{20}-C_{30}) fatty acids, alcohols, hydroxyfatty acids and dicarboxylic acids.

There are many ways in which lignification/suberization might inhibit microbial diseases: i) host cell walls at that region of invasion might be made more resistant to mechanical penetration, ii) plant cell walls at the point of attack might be rendered resistant to dissolution by microbial cell wall degrading enzymes, iii) "ligno-suberin" might restrict the diffusion of toxins and enzymes from a pathogen to the host, iv) similarly "ligno-suberin" might restrict plant-produced antimicrobial compounds (e.g., phytoalexins) from diffusing out of the diseased area effecting local accumulations to antimicrobial concentrations, v) lignification/suberization may form a type of insoluble water barrier between healthy plant tissue and the infected plant tissue, thus leading to desiccation of the infected area and consequent inhibition of microbial growth, vi) low molecular-weight phenolic precursors of lignin and/or suberin and free radicals produced during polymerization might in themselves be antimicrobial, thus there is an antibiotic potential associated with the formation of "ligno-suberin" and vii) pathogen cell wall "hardening" might occur due to lignification and the plasticity necessary for microbial growth might be reduced (mycelium can absorb lignin and fungal and bacterial cell walls contain chitin, cellulose and/or hydroxyproline-rich glycoproteins which can serve as matrices for the polymerization of lignin precursors (Aist, 1985; Bell *et al.*, 1981; Crute *et al.*, 1985; Vance *et al.*, 1980).

I.3.4 Phytoalexins

The foremost studied and perhaps the most important active plant defense mechanism is the production of antibiotic compounds called phytoalexins. Phytoalexins are generally defined as low molecular-weight antimicrobial compounds that are synthesized by and accumulate in plant cells after exposure to microbes (Dixon, 1986). The ability of higher plants to synthesize and accumulate phytoalexins is widespread, if not ubiquitous, being present in monocotyledonous and dicotyledonous plants (Darvill and Albersheim, 1984). An antimicrobial defense function for these compounds has been proven by the inhibitory and toxic effect that purified phytoalexin compounds exert on the growth of microbes *in vitro* (Smith, 1982). The fact that phytoalexins are absent in healthy plants and the ability of plants to synthesize and accumulate these compounds at the site of microbial infection, suggests the role these compounds may perform in active plant defense. A number of studies have provided evidence that phytoalexins accumulate at the site of infection following ingress or exposure to microbes, quickly enough and in sufficiently high concentrations to inhibit the growth of both fungi and bacteria (Smith, 1982; Darvill and Albersheim, 1984). The antimicrobial compounds produced by plants after infection include a variety of chemical compounds of different chemical structure: isoflavonoids, flavonoids, dihydroxyphenanthrenes, stilbenes, coumarins, isocoumarins, terpenoids, furanoacetylenes, polyacetylenes and polyenes. Nearly all species in the *Leguminosae*, *Solanaceae* and *Mabraceae* synthesize phytoalexins in association with a necrogenic resistance to phytopathogens (Darvill and Albersheim, 1984).

Keen (1982) states in one review that there is substantial evidence from several gene-for-gene systems, that phytoalexins accumulate to high concentrations after inoculation of plants with the avirulent, but not the virulent parasite races and he cites 16 examples. In addition, the consistent correlation of high phytoalexin synthesis only in the incompatible host reactions in these systems, strongly supports their role as mediators of disease resistance. For example, phytoalexin accumulation was determined in both resistant and sensitive soybean cultivars infected with *Phytopthora megasperma* f. sp.

glycinea (Yoshikawa *et al.*, 1978). The concentration of the phytoalexin glyceollin, was determined in 0.25 mm sections around the invading hypha. In the incompatible reaction, concentrations of glyceollin capable of inhibiting mycelial growth by 90%, *in vitro*, were present near the hyphal tips 8 hrs after inoculation, at which time, glyceollin could hardly be detected in the compatible reaction.

Phytoalexin accumulation has been detected during the hypersensitive response (HR) in several associations between plants and bacteria. In a detailed examination of the plant-pathogen interaction between soybean and *Pseudomonas syringae* pathovar (pv.) *glycinea*, hypersensitive necrosis was first observed 9 hrs after bacterial inoculation into soybean leaves. Glyceollin was first detectable in leaf extracts at 16 hrs post-inoculation and inhibition of bacterial multiplication was apparent by 30 hrs post-inoculation, when glyceollin concentrations were maximal (Holliday and Keen, 1982).

In another example, the rate of accumulation of phytoalexins in broad bean epidermis tissue at the site of infection by *Botrytis fabae* (a fungal pathogen) and the closely-related, but nonpathogen, *B. cinera*, was studied (Crute *et al.*, 1985). In the case of the nonpathogen, *B. cinera*, wyerone and wyeronic acid began to accumulate 8 hrs post-inoculation and increased to a maximum at 22 hrs; this increase correlated closely with the cessation of fungal growth. Infection by the pathogen, *B. fabae*, was characterized by rapid penetration and killing of host cells, before they could accumulate sufficient phytoalexin to stop fungal growth (Rossall *et al.*, 1980).

Phytoalexins have been studied mostly in *Leguminosae* and in the *Solanaceae*, but recently a novel phytoalexin has been discovered in the small cruciferous plant *Arabidopsis thaliana* (Tsuji *et al.*, 1992). Inoculation of leaves of *A. thaliana* with the wheat pathogen, *P. syringae* pv. *syringae*, resulted in the expression of hypersensitive necrosis and in the accumulation of a phytoalexin. This phytoalexin accumulated rapidly in leaves within 12 hrs post-inoculation with *P. syringae* pv. *syringae* and was associated with the cell collapse that occurred during the HR. The *in vitro* growth of *P. syringae* pv. *syringae* was inhibited by the purified phytoalexin compound, and the *in planta* growth of *P. syringae* pv. *syringae* and the accumulation of the *Arabidopsis* phytoalexin were negatively associated. No phytoalexin accumulation was detected after infiltration of leaves with a mutant *P. syringae* (*hrp*⁻) strain, deficient in the ability to cause disease or elicit the HR, or with the cauliflower pathogen *Xanthomonas campestris* pv. *campestris*. These data indicate that a phytoalexin plays a role in the defense mechanism of *Arabidopsis* plants against specific bacterial pathogens. Also, the data indicates that the phenylpropanoid isoflavonoid and the flavonoid phytoalexins, which are common in *Leguminosae* and *Solanaceae*, are probably not active components of the *Arabidopsis* phytoalexin arsenal. Rather a novel family of sulphur-containing indole-derived compounds appear to be the principal phytoalexins in *Arabidopsis* and other crucifers (Browne *et al.*, 1991; Jejelowo *et al.*, 1991; Tsuji *et al.*, 1992; Gross, 1993; Tsuji *et al.*, 1993).

The mechanism by which phytoalexins act to inhibit microbial cell growth has not been studied in great length (Darvill and Albersheim, 1984). Phytoalexins have definite antimicrobial properties *in vitro*, however, toxic activity across much of the biological spectrum, including eukaryotic cells (plant and mammalian), has been demonstrated (Smith, 1982). At physiological concentrations phytoalexins often have a static effect rather than a toxic effect on microbes (Darvill and Albersheim, 1984). Little can be stated concerning the molecular features which confer antimicrobial activity. The lipophilic nature of these molecules seems to be a common feature which possibly permits effective penetration of microbial cytoplasmic membranes disrupting membrane integrity. Common cytological and physiological effects of phytoalexins verify the likelihood that membrane inactivation is an important aspect of the mode of action. Leaking (observed by increases in conductivity), swelling and bursting of mammalian cells and fungal/bacterial protoplasts and substantial losses in dry cell-weight of treated cells are all observed shortly after exposure to phytoalexins (Smith, 1982). The rapidity of toxic effects on the cells rules out, as the primary site of action, inhibition of DNA, RNA, or protein synthesis.

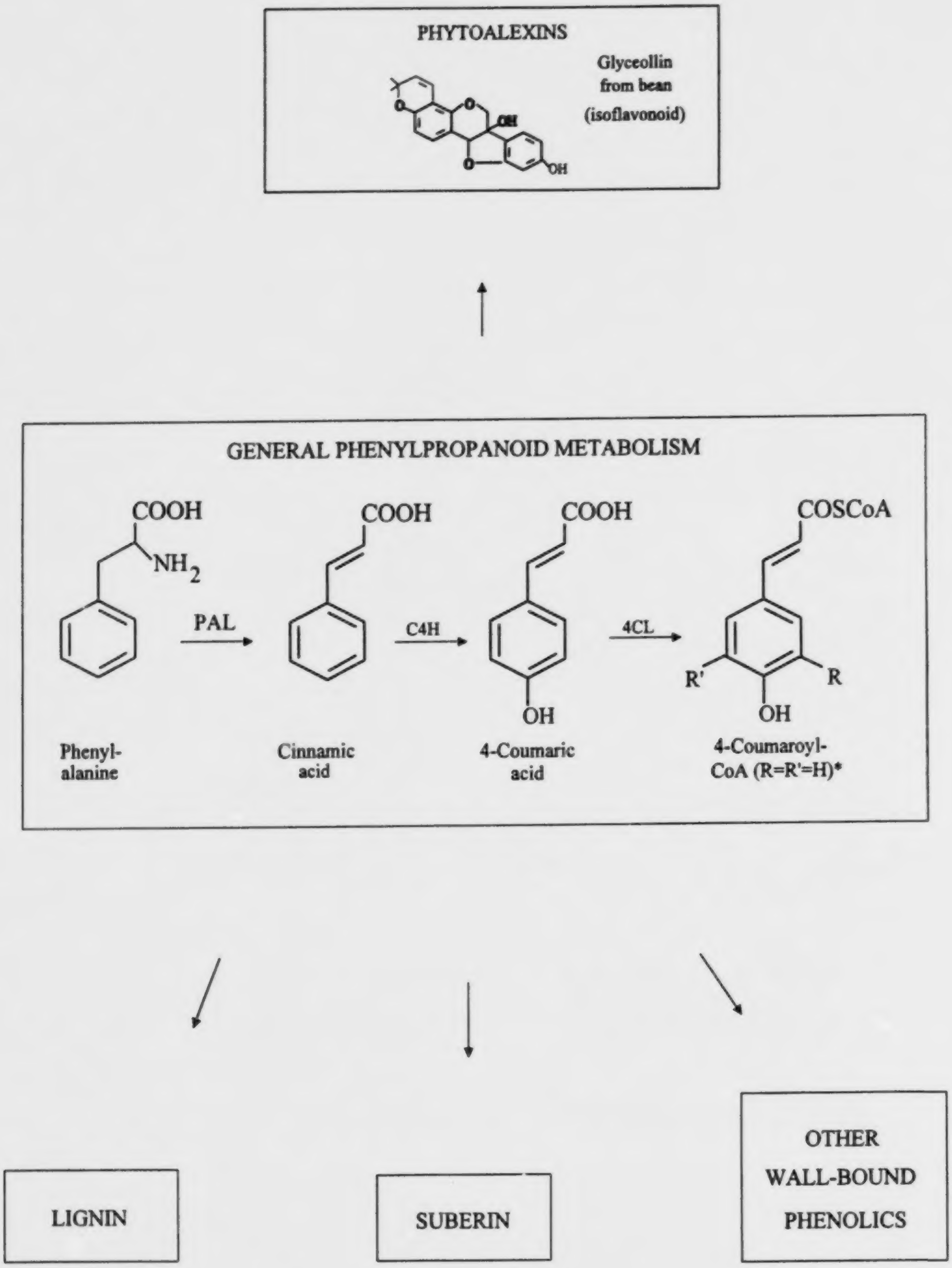
Since phytoalexins come from a wide variety of chemical classes with corresponding structural differences, it might be expected that different compounds could possess specific targets and have different modes of action. Glyceollin, a pterocarpan phytoalexin, has been shown to specifically inhibit site I of electron transport between NADH dehydrogenase and coenzyme Q in mitochondria (Boydston *et al.*, 1983). Yet evidence provided by Weinstein and Albersheim (1983) demonstrated that glycinol, another pterocarpan phytoalexin, induced its bacteriostatic effects by intercalating and thus damaging the overall stability of the cytoplasmic membrane. Another study showed that an induced tolerance in the fungus *Nectria haemotococca* to pterocarpan phytoalexins derived from pea, was due to a modification of the fungal cytoplasmic membrane (Denny and Van Etten, 1983). Similar toxic effects are observed in a variety of cells, therefore, a general type of membrane perturbation causing membrane dysfunction appears to be the mode of phytoalexin activity (Smith, 1982). Toxic effects are also observed in plant cells, which appears to establish a link between phytoalexin accumulation and hypersensitive necrosis. Phytoalexins are synthesized in live cells, but cell necrosis follows very quickly, possibly within minutes. Thus, peak concentrations of phytoalexins in tissue almost always coincide with visible necrosis (Bell, 1981).

In many plants belonging to *Leguminosae* and the *Solanaceae*, phytoalexin production may be sufficient to account for resistance to some potential pathogens; phtyoalexin accumulation is invariably associated with plant cell death and necrosis, although it is not easy to determine if the hypersensitive necrosis causes phytoalexin accumulation, or *vice versa* (Dixon, 1986). Studies on potato using 12 different cultivars exhibiting different degrees of resistance to *P. infestans*, indicated that the degree of resistance depended primarily on the rapidity of occurrence of hypersensitivity, and the associated accumulation of the phytoalexin rishitin (Doke, 1982). It is likely that plant disease resistance requires rapid HR necrosis and the concerted and timely activation of many defense mechanisms, such as phytoalexin production and lignification. In combination, these defence responses probably exert a synergistic effect inhibiting the growth of microbial pathogens.

I.4 PHENYLPROPANOID METABOLISM

Of direct relevance to this thesis is the control of the phenylpropanoid pathway. The many plant-specific phenylpropanoid branch pathways and the corresponding functional diversity of their products have long attracted attention in plant physiology. In recent years, the specific transcriptional inducibility of genes for this pathway has become the focus of much research. The functions of phenylpropanoid derivatives are as diverse as their structural variations. Phenylpropanoids serve as flower pigments, UV-light protectants, insect repellents and signal molecules in plant-microbe interactions (Hahlbrock and Scheel, 1989). Phenylpropanoid molecules also function as complex polymer constituents of surface and support structures such as lignin and as suberin, other cell wall components and as phytoalexins. The key enzyme in the phenylpropanoid pathway is L-phenylalanine ammonia-lyase (PAL). PAL is responsible for the conversion of L-phenylalanine to cinnamic acid and NH_4^+, which is the first step in the core reactions of the phenylpropanoid metabolism. The PAL genes are under a multifaceted control system, which includes induction by mechanical wounding, UV-light exposure and microbial challenge (Hahlbrock and Scheel, 1989). PAL genes are also controlled in both a developmental and tissue-specific fashion. This feature of multifaceted PAL gene regulation is not unusual, since nearly all plant defense genes also have developmentally regulated cell-type specific functions during plant growth (Kiedrowski *et al.*, 1992). PAL belongs to small multigene families in most plants. Multigene families encoding PAL have been reported in parsley, rice, potato and bean. *Arabidopsis* is specifically thought to contain at least four PAL genes. Nothing indicates that these genes are linked within the plant genome. The multiplicity of PAL genes in the plant genome reflects the varied role of phenylpropanoids in plant metabolism. It is believed that different PAL genes, encoding different isoforms of PAL enzyme, are differentially induced depending on environmental challenge (i.e., microbial infection), tissue-specific, or developmental demands of the plant (Shufflebottom *et al.*, 1993; Hahlbrock and Scheel, 1989). The major influence on PAL activity appears to be *de novo* synthesis of the enzyme. PAL genes and cDNA clones have been isolated from bean (Liang *et al.*, 1989), potato (Joos and Hahlbrock, 1992), rice (Minami *et al.*, 1989), parsley (Lois *et al.*, 1989) and recently from *Arabidopsis* (Ohl *et al.*, 1990). Use of these clones in several Northern hybridization studies, using different whole plant or plant cell suspension systems, has demonstrated increases in the steady-state level of PAL mRNA in response to several environmental challenges; most commonly microbial inoculation, exposure to small fungi-derived oligosaccharide cell wall fragments, or UV-light exposure. In several instances, Northern hybridization studies have demonstrated that increases of PAL enzyme activity, which are followed by the accumulation of phenylpropanoid

Figure 1: Scheme illustrating the flux of phenylalanine-derived intermediates from the core reactions of general phenylpropanoid metabolism to some of the major branch pathways. The asterisk indicates the possible introduction of structural variations, predominantly in the 3 and 5 positions (R, R'), of 4-coumaroyl-CoA, catalyzed by enzymatic reactions, either preceding, or following CoA thiol ester formation by 4CL. PAL, C4H and 4CL are abbreviations for the enzymes phenylalanine ammonia-lyase, cinnamate4-hydroxylase and 4-coumarate: CoA ligase (adapted from Hahlbrock and Scheel, 1989).


PHYTOALEXINS
Glyceollin from bean (isoflavonoid)
OH
OH
O
GENERAL PHENYLPROPANOID METABOLISM
COOH
NH2
PAL
C4H
4CL
COSCoA
R'
R
OH
Phenyl-alanine
Cinnamic acid
4-Coumaric acid
4-Coumaroyl-CoA (R=R'=H)*
LIGNIN
SUBERIN
OTHER WALL-BOUND PHENOLICS

metabolites, are invariably preempted by the transcriptional activation of the PAL genes. Parsley cell suspension cultures showed increases in PAL enzyme activity of 5-7-fold at 12 hrs post-exposure and high levels of the phytoalexin, medicarpin, were detected 24 hrs after exposure to a fungal cell wall-derived elicitor (Dalkin *et al.*, 1990). Also parsley cell suspension cultures, when treated with an elicitor preparation from the fungus *P. megasperma* f. sp. *glycinea*, showed increased PAL mRNA and an accumulation of furanocoumarin phytoalexins. Using French bean cell suspension cultures treated with elicitor, or excised hypocotyls inoculated with fungal spore suspensions, the isoflavonoid phytoalexin compounds phaseollin and kievitone were observed to accumulate in French bean; transcriptional activation of the genes encoding PAL were also concomitantly noted (Hahlbrock *et al.*, 1981; Kuhn *et al.*, 1984). In a similiar system using French bean cell suspension cultures, a cDNA clone of the cinnamyl alcohol-dehydrogenase gene was also isolated; RNA hybridisation studies using this clone demonstrated that lignification was induced concomitantly with phytoalexin production in that system (Lamb *et al.*, 1986). Also in *Raphanus japonica* PAL activity and lignification were found to increase following infection with *Peronospora parasitica* and *Alternaria japonica* (Collinge and Slusarenko, 1987). As cited previously, lignification and elevated PAL activity were recorded from resistant, but not susceptible discs, of different potato tuber varieties following challenge by races of *P. infestans* (Henderson and Friend, 1979). The importance of PAL and its defense related phenylpropanoid end-products was demonstrated by the treatment of soybean plants with α-aminooxy-β-phenyl-propionic acid, a potent inhibitor of PAL *in vivo*. Resistant soybean plants which were treated in this way became highly susceptible to a previously incompatible race of *P. megasperma* f. sp. *glycinea* (Dixon, 1986).

Typically, transcriptional induction studies show maximum levels of PAL mRNA are achieved 4-10 hrs after exposure to microbes, however, the duration of the induction period varies with the experimental conditions and the particular plant-microbe or plant-elicitor combination used. Increases in PAL enzyme activity characteristically follow shortly afterwards (several hours after maximal PAL mRNA levels), with increases in phenylpropanoid end products such as lignin or phytoalexins attaining maximal levels a minimum of several hours after maximal enzyme activities. Thus, in the plant resistance mechanism there exists: i) a determinative phase or induction phase occurring shortly after exposure to a microbe, which is typified by the transcriptional activation of defense genes and ii) an expressive phase or latent period, characterized by mRNA translation and the synthesis and accumulation of antimicrobial compounds (Keen and Bruegger, 1977; Sigee, 1993). In many cases mRNA accumulation, for a given plant defense gene, is very rapid during a resistance reaction, but is markedly slower in susceptible reactions; differences in magnitude between levels of accumulation in resistant and susceptible plant genotypes are also often observed, but not universally (Kiedrowski *et al.*, 1992).

More recently, experiments using *Arabidopsis* have described 8-fold increases in PAL mRNA in leaves, 6 hrs after inoculation with an avirulent *P. syringae*, in comparison with leaves inoculated with an isogenic, but virulent *P. syringae* strain. Interestingly, the chalcone synthase gene was not induced, leading Dong *et al.* (1991) to conclude that flavonoid phytoalexins are not likely produced in *Arabidopsis* as a defense response. This supposition agrees with the previously cited work of Tsuji *et al.* (1992), who isolated a indole-derived phytoalexin rather than a flavonoid phytoalexin. Previously Davis and Ausubel (1989) found that two other phenylpropanoid biosynthetic enzymes, caffeic acid *O*-methyl transferase and peroxidase, which are specifically involved in lignin biosynthesis, were induced, suggesting that the rapid PAL gene expression is ultimately a strategy for supplying phenolic precursor for the production of lignin or ligno-suberin in infected plants.

I.5 GENERAL MICROBIAL PROPERTIES WHICH CONFER VIRULENCE

From the microbial side, phytopathogenic microorganisms generally fall into two broad categories with respect to their abilities to pathogenize plants. Association with a plant-host species exhibits a range of specificities dependent upon the strategy of the pathogen (Lamb *et al.*, 1989). One type of pathogen is the unspecialized "thug" which is necrolytic and damages the host through the production of toxins and enzymes. In contrast, "con-men" grow biotrophically avoiding serious host-damage, at least in the early stages and unlike "thugs", do not activate host defenses non-specifically. For these specialized pathogens, without their avoidance mechanisms, host defences, if induced, are usually effective. Following attempted infection by the pathogen, in these specialized cultivar/race-specific plant-pathogen interactions, molecular signals determine whether the interaction will be compatible or incompatible.

The functions of genes that determine the virulence/avirulence of plant pathogens and the concomitant susceptibility/resistance of plants to infection, with few exceptions, are only understood in a rudimentary way (Panopoulos and Peet, 1985). Successful infection by microbial pathogens requires surface attachment, degradation of host chemical and physical barriers, inactivation of host plant active defense responses and sometime includes the production of toxins. Overall it has been estimated that at least several dozen to one hundred genes are involved in general bacterial pathogenicity (Panopoulos and Peet, 1985). At least three broad classes of genes have been classified in determining bacterial pathogenesis. The first class of genes includes those genes which code for general virulence factors and pathogenesis mechanisms and therefore positively regulate virulence. Plant pathogens increase their ability to colonize and damage host-plant tissue by expressing several virulence mechanisms. Some of these general mechanisms include the production of: exopolysaccharides (EPS), cellulolytic enzymes, toxins and plant growth regulators (Chaterjee and Vidover, 1986; Keen, 1992; Sigee, 1993). Any of the genes which encode the forementioned mechanisms can be loosely classified as virulence genes.

Various universal roles for EPS in bacterial pathogenesis have been suggested including the inducing and maintenance of water-soaking, restriction of water movement, prevention of recognition of bacteria by the infected plant and changes in bacterial carbohydrate metabolism (Chaterjee and Vidover, 1986; Sigee, 1993). The production and secretion of a wide range of enzymes, primarily cellulases, pectolytic enzymes and proteases, are also a general virulence feature of phytopathogenic microbes, especially the soft rot bacteria (Sigee, 1993). These enzymes act to degrade plant cell walls causing plant cell lysis, resulting in tissue disintegration and releasing nutrients that encourage microbial growth. These enzymes constitute an important virulence feature. Phytopathogens that are deficient in their ability to synthesize and secrete extracellular degradative enzymes generally have a reduced virulence (Pirhonen *et al.*, 1993). Phtyopathogens can also produce low molecular-weight phytotoxins which are not host-specific, but are in general important virulence factors (Sigee, 1993; Goto, 1992). Infiltration of non-toxin producing strains, into host-plants, has shown that in most cases toxins are not essential for pathogenesis *in planta*, though they do contribute to its growth and spread. Mutants deficient in the ability to produce toxins often achieve lower population levels in host-tissue compared to the wild-type and produce less pronounced disease symptoms. Remote host-cell dysfunction may be brought about by the secretion of toxins, causing plant cell death and releasing water and nutrients at some distance, which probably encourage pathogen growth and spread (Sigee, 1993). Phytopathogens certainly possess several virulence mechanisms which promote their growth and spread in plants and contribute to their pathogenicity. However, the question still remains: what determines host-range or specific pathogenicity? The remaining two classes of microbial genes involved in plant pathogenesis and specifically have been identified as being vital in restricting host-range are: i) the fungal/bacterial avirulence genes (*avr*) and ii) the bacterial hypersensitive reaction and pathogenicity (*hrp*) genes. It is the presence or absence of these specific microbial genes, in relation to the complementary plant resistance genes, which determines race/cultivar specificity in plant-pathogen interactions. Genetic analysis of plant-microbe interactions has demonstrated that race-specific resistance is specified by the presence of single avirulence genes in the microbe that correspond to single resistance genes in the plant.

I.6 GENETIC DETERMINANTS SPECIFYING HOST-RANGE OF PLANT -PATHOGEN INTERACTIONS

I.6.1 Basic Concepts of Host-Specificity in Plant-Pathogen Interactions

Plants, unlike mammals which have a complicated multifaceted immune system, use an altogether different strategy to combat infection. This strategy, which in effect completely severs and results in the deletion of the infected areas, is hardly one which would work for animals. Nonetheless, in plants the death of cells in and around the region of infection resulting in dry cell necrosis proves to be an efficient defense mechanism isolating the pathogen and preventing further colonization of healthy plant tissues (Pryor, 1987). This "overreaction" or hypersensitive response (HR) of resistant plant cells, is the predominant mode of expression of a single major resistance gene. HR involves relatively rapid effects on plant cell membranes, followed by the rapid necrosis of plant cells in the vicinity of the invading pathogen (Mathieu *et al.*, 1991; Gabriel *et al.*, 1988). Plant cell necrosis *per se* may not directly contribute to resistance since the actual inhibition of pathogen development and spread often occurs later (Keen, 1992). This resistance frequently correlates closely with the formation of phytoalexins and the erection of structural barriers such as lignin, in and around the infection site (Keen, 1992; Collinge and Slusarenko, 1987). The expression of many plant defense genes not only those involved in phytoalexin biosynthesis or lignin deposition are activated; for example other genes which are induced are glucanases, chitinases and HRGPs (Keen, 1992; Collinge and Slusarenko, 1987). Presumably, specific plant resistance requires both HR-necrosis and the *de novo* expression of numerous defense genes, which may act synergistically to limit the growth of microbial pathogens. The most interesting genetic feature of specific HR resistance is what is known as the gene-for-gene hypothesis. Flor (1955) described a gene-for-gene relationship between a host's resistance gene (R) and a pathogens avirulence gene (A) as follows: the host possesses a specific R allele which can recognize a specific pathogen's A allele and the incompatibility of an interaction occurs only when a matching specific A/R combination is present.

In several host-pathogen interactions that have been studied in detail, genetic analysis has revealed a gene-for-gene correspondence between a particular plant resistance gene (R), which "recognizes" a pathogen's avirulence gene (A), causing the plant to mount a localized HR; this hypothesis is generally accepted as universally valid in predicting and describing the race/cultivar specificity in plant-pathogen interactions. Generally plant parasitic microbes have the capability to induce the HR while saprophytes do not; in addition those bacterial parasites which induce the HR, tend to be host-specific, while those parasites with an extensive host-range tend not to be capable of inducing the HR (Klement, 1964 and 1982). Microbial populations in compatible interactions increase dramatically within 48 hrs of inoculation and final cell numbers can increase 10^5-fold over initial inoculum levels. On non-host plants, the incompatible interaction is often correlated with the elicitation of the hypersensitive reaction and microbial growth within the intercellular spaces of incompatible leaf tissue is limited, with the final populations either remaining unchanged, or increasing only 10-100-fold within the first 48 hrs (Willis *et al.*, 1991). The introduction of nonpathogenic organisms, such as *Escherichia coli* or *P. flourescens*, does not result in the appearance of an HR and these bacteria do not multiply in the plant tissue.

If a plant's resistance gene(s) "recognizes" a specific pathovar's avirulence gene(s), then the plant exhibits a localized HR. Another cultivar of the same plant species lacking that resistance gene will be susceptible to the pathogen. Similarly, another pathovar of the same bacterial species lacking the avirulence gene will be pathogenic; thus the cultivar/race specific phenomenon of plant disease resistance. The model predicts that the transfer of an avirulence gene to a virulent pathogen will make it avirulent on plants carrying the corresponding resistance genes and that mutations in the microbial avirulence gene will result in virulence. Conversely, from the plant side, it is predicted that the transfer of a resistance gene to a closely related plant cultivar, lacking that resistance gene, will result in resistance to pathovars carrying the corresponding avirulence gene; mutations in that resistance gene will result in loss of resistance. Absence or inactivation of either member of this gene pair results in loss of resistance (Shield and Stratford, 1993). The molecular cloning and transfer of several bacterial avirulence genes in recent years, that are capable of imparting an avirulent phenotype when transferred to an otherwise isogenic virulent strain, has granted the gene-for-gene hypothesis definitive proof.

I.6.2 Microbial Avirulence Genes

Microbial races are functionally defined by the collection of avirulence genes they contain (Keen, 1992). It is the assorted avirulence genes that a plant pathogen possesses which appears to determine the pathogen's host-range. The *P. syringae* taxon includes some 40 pathovar designations, which differ in their respective plant-hosts at the level of plant species and which are pathogenic on many agronomically important crops; another example is the *Xanthomonas campestris* taxon which contains over 100 defined pathovars (Long and Staskawicz, 1993).

The first avirulence gene was cloned from *P. syringae* pv. *glycinea* race 6 by Staskawicz *et al.* (1984). This bacterial isolate was incompatible on soybean cultivars Acme, Chippeway, Harsoy and Peking. A gene was cloned from *P. syringae* pv. *glycinea* race 6, which when transferred to *P. syringae* pv. *glycinea* races 1, 4 and 5, changed the cultivar specificity of these races to that of race 6; the transformed bacteria now caused HR necrosis when inoculated onto the said soybean cultivars. The gene was designated *avr A*, was sequenced and was found to code for a 100 kDa protein. The Avr A protein contained no significant hydrophobic domains and possessed no leader-peptide secretion-sequence that would be expected if it were membrane associated, or exported extracellularly (Napoli and Staskiwicz, 1987). Thus initial confirmation of the prediction of Flor's gene-for-gene hypothesis of a dominant avirulent gene was substantiated and the transfer of an avirulence gene did allow particular genotypes of soybean to recognize and mount a defense response to phytopathogenic bacteria which possessed this gene.

Shortly thereafter, two other avirulence genes from a single isolate of *P. syringae* pv. *glycinea* race 0 were cloned. The *avr B* and *avr C* genes were subsequently sequenced and were found to encode for single protein products of 36 and 39 kDa respectively; like *avr A* they did not possess a signal secretion sequence or any hydrophobic domains (Tamaki *et al.*, 1988). They were generally hydrophillic and they possessed no homology to any protein in the databases. However, Avr B and Avr C proteins displayed considerable homology to one another and it was shown that the nonhomologous central domain was involved in conferring cultivar specificity. The Avr B and Avr C proteins have been produced in large amounts in *E. coli*; the purified proteins did not elicit the HR when infiltrated into soybean leaves and it remains unclear how these proteins interact with a plant recognition factor (i.e., the complementary plant resistance gene product), to elicit HR.

Several cosmid clones from a DNA cosmid library of *P. syringae* pv. *tomato* were isolated which upon introduction into *P. syringae* pv. *glycinea* caused them to elicit a HR in certain, but not in all soybean cultivars (Koyabashi *et al.*, 1989). Thus, *P. syringae* pv. *tomato* (a tomato pathogen) contains avirulence genes that elicit the HR resistance in soybean, in a race-specific manner, when they are transferred to a closely related soybean-virulent bacterium. Of significant interest was the discovery that one of the *P. syringae* pv. *tomato* avirulence genes was found to be identical to the *avr A* gene first isolated from *P. syringae* pv. *glycinea* race 6 (Napoli and Staskawicz, 1987). The *avr A* gene was later found to be present in all of 14 different isolates of *P. syringae* pv. *tomato*. Avirulence genes are, therefore, not unique to a single race or pathovar, but may be common to several pathovars; consequently a single avirulence gene might be able to elicit the HR in more than one host plant and hence be important in determining pathogen specificity above the race/cultivar level.

Koyabashi *et al.* (1988) also addressed this question by introducing the cloned *avr A* gene from *P. syringae* pv. *tomato* into several other *P. syringae* pathovars and inoculating the transformants onto their normally susceptible hosts. Introduction of the *avr A* gene did not effect the pathogenicity of *P. syringae* pathovars *lachrymus*, *phaseolicola*, or *pisi* on their hosts, but *P. syringae* pv. *tabaci* transformed with *avr A* elicited an HR on tobacco plants, rather than precipitate their usual disease symptoms.

Similar phenomenon has also been demonstrated in the *Xanthomonas* taxon. *X. campestris* pv. *vesicatoria* is the causal agent of leaf-spot disease on pepper and tomato. In nonhost plants such as soybean, alfalfa and cotton, *X. campestris* pv. *vesicatoria* is unable to cause disease, but instead it induces an HR necrosis (Whalen *et al.*, 1988). A cosmid clone from *X. campestris* pv. *vesicatoria* converted numerous *X. campestris* pathovars to avirulence on their host. When *X. campestris* pathovars *glycinea* (soybean), *vignicola* (alfalfa), *malvacearum* (cotton) and *holicola* (maize), were transformed with the cosmid clone they all became avirulent on their normally susceptible hosts. However, pathovars *vitian* and *campestris* were unaffected and still remained virulent on their corresponding hosts. Furthermore, the HR resistant phenotype in bean, to *X. campestris* pathovars carrying the *avrRxv* gene, segregated as a single, semi-dominant, Mendelian gene. Resistance genes might be common to different plant species and a single avirulence gene may be able to elicit the HR in more than one host plant; therefore, there is proof which suggests that avirulence genes and plant resistance genes are likely to be important in determining pathogen specificity above the race/cultivar level. Since the initial discovery and cloning of the first avirulence gene, over 30 different avirulence genes from various phtyopathogenic bacteria have been successfully isolated.

Of direct relevance to this thesis is the recent cloning of *avrRpt2* from *P. syringae* pv. *tomato* (*Pst*); *Pst* is avirulent on several ecotypes of *A. thaliana* and elicits a strong HR when infiltrated in *Arabidopsis* leaves (Dong *et al.*, 1991). The transfer of *avrRpt2* into *P. syringae* pv. *maculicola*, which is virulent on *Arabidopsis*, resulted in an avirulent phenotype and caused the *Arabidopsis* plants to elicit an HR at the infiltration site. The authors identified a rapid, large, specific accumulation of PAL mRNA approximately 6 hrs after infiltration of the avirulent *P. syringae* pv. *tomato* or *P. syringae* pv. *maculicola* (*avrRpt2* containing) cells; in contrast, infiltration with the virulent *P. syringae* pv. *maculicola* cells resulted in only a modest increase of PAL mRNA. This was the first study to demonstrate a direct link between the specific induction of a plant defense gene and the HR-necrosis in response to a specific bacterial avirulence gene.

The obvious inference is that microbial avirulence genes encode determinants for host recognition by way of a specific plant resistance gene, but the question remains: what are the bacterial determinants, could they be surface proteins and/or low molecular-weight signals? Recent results from two separate systems imply that it can work in at least two completely different ways (Long and Staskawicz, 1993). The two main hypotheses at present are that avirulence gene products function in an indirect manner either controlling components related to the surface architecture of the bacterium, or participate in the production of diffusible cultivar-specific elicitors. The *avr B3* gene from *X. campestris* pv. *vesicatoria* race1 codes for a 122 kDa protein with 17.5 direct repeats. The repeat-unit is highly conserved and consists of 34 amino acid (Bonas *et al.*, 1989). The significance of this feature is unclear, however, other proteins carrying similar direct repeats such as *Pseudomonas* ice-nucleation protein, *E. coli* haemolysin, erythrocyte spectrin protein and extensin protein from plant cell walls, all are associated with the outer membrane or with structural components of the cell. Secondary structure for the region spanning the repeats suggests a possible localization of the protein in the cell wall (Bonas *et al.*, 1989). The *avr B3* gene belongs to a family of over 16 avirulence genes borne by diverse pathogens and each one specifies a unique pattern of host resistance specificity (Knoop *et al.*, 1991). Changing the number of the internal repeats by gene deletion resulted in new avirulence gene activities, which specified completely new patterns of resistance in plant-hosts (Herbers *et al.*, 1992). This suggests that these avirulence proteins may be surface structural components and may interact directly with the plant host-cells to elicit the HR. The second mechanism involves the secretion of low molecular-weight chemical signals or elicitors. Evidence for the involvement of an elicitor comes from recent studies of the *avr D* gene from *P. syringae* pv. *tomato* (Kobayashi *et al.*, 1990). The cloned *avr D* gene encodes for a 34 kDa protein of unknown function. Its expression in several gram negative bacteria, including *E. coli*, caused them to secrete, a low molecular-weight elicitor molecule that was recognized by soybean plants possessing the *Rpg4* resistance gene (Keen *et al.*, 1990). Because the gene is functional in *E. coli*, the elicitor molecule must be derived from common bacterial metabolites. The avr D elicitor has recently been isolated and was determined to be to two similiar gamma lactones (Keen *et al.*, 1993). These molecules were not encountered previously and were given the trivial names syringolide 1 and 2. The structures of the avr D elicitors suggest that they may be formed biosynthetically from the result of the condensation of a β-keto fatty acid (either β-ketooctonoic or β-ketodecanoic acid) with the sugar xylose. They have some similarity to the butryolactones isolated from *Streptomyces* sp. and to the homoserine-lactones isolated from *Erwinia* sp. and *Agrobacterium* sp. In these genera these molecules function in bacterial cell-to-cell communication; of sporulation in *Streptomyces* and in the activation of virulence determinants in *Erwinia* and *Agrobacterium* (Horinoudu and Beppu, 1992; Pirhonen *et al.*, 1993; Zhang *et al.*, 1993). The avr D elicitor molecules of *Pseudomonas* may function in a similar fashion to the *Erwinia* auto-inducer molecule in controlling the production of exoenzyme virulence determinants *in planta*. Planst may have evolved a mechanism to detect the same vital signal molecule involved in bacterial cell-to-cell control of virulence and thereby elicit a defense response when exposed to virulent phytopathogenic bacteria synthesizing this molecule.

Avirulence genes can be found on plasmids and they are often chromosomally located, although they do not appear to be linked to the *hrp* genes (Panopoulos and Peet, 1985). Avirulence genes act negatively since they reduce host-range, thus, the retention of an avirulence gene confers a selective disadvantage to the pathogenic organism growing on cultivars containing the complementary resistance gene; the question consequently arises: why would avirulence genes be retained in bacterial populations? Avirulence genes are not required for growth *in vitro* and do not appear to be required for pathogenesis *in planta*, however, they are two recent examples were avirulence genes do in fact appear to be required for full virulence *in planta*. It has been discovered that some avirulence genes are highly conserved in corresponding pathovars. For instance *avr Bs2* is highly conserved among many strains of *X. campestris* pv. *vesicatoria* and among 20 other pathovars of *X. campestris* (Kearney and Staskawicz, 1990). Moreover, it was found that *avr Bs2* was in fact needed for full virulence of the pathogen on its susceptible host. An *avr Bs2*-minus mutant, although still able to pathogenize and multiply on a susceptible host-plant, showed significantly lower bacterial growth and cell numbers *in planta*, than the identical wild-type strain. Similarly, a *X. campestris* pv. *citri* gene was cloned that permitted another citrus pathogen, *X. campestris* pv. *citrumelo*, to form the canker-like lesions on citrus leaves which are normally unique to the disease caused by *X. campestris* pv.

citri. This gene designated *pth A* bestows pathogenesis, yet it has a high degree of homology to the bacterial avirulence gene *avr Bs3* (referred to earlier) and when introduced into other bacterial pathogens, such as *X. campestris* pv. *phaseoli* and *X. campestris* pv. *malvacearum*, caused them to induce the HR and on their normally susceptible hosts. These are the only two documented examples of avirulence genes in bacteria that can cause normally susceptible plants to induce a defensive response in some cases and also are capable of conferring virulence in others (Long and Staskawicz, 1993).

The same gene-for-gene complementarity specifying race/cultivar compatibility exists between isogenic races of fungi and congenic plant cultivars (Oku, 1992; Oliver, 1992). Although, the genetic manipulation of fungi is far less advanced than bacteria and to date only one fungal avirulence gene has been cloned. A small 28 amino acid peptide was produced in tomato leaves infected by *Cladosporium fulvum* isolates carrying the putative *avr9* avirulence gene. The peptide caused HR necrosis and resulted in *C. fulvum* avirulence on tomato cultivars carrying the putative *Cf9* resistance gene. Following the isolation, purification and sequencing of the peptide, an oligonucleotide probe was constructed and used to screen cDNA and genomic clones isolated from libraries of *C. fulvum* biotype *avr9*. A clone was isolated and the corresponding *avr 9* avirulence gene was sequenced and found to encode for a 63 amino acid precursor protein. Transformation to the *avr 9* gene into pathogenic *C. fulvum* isolates, lacking the *avr 9* gene, caused the fungi to elicit an HR when inoculated onto *Cf9*-tomato plants, thus, converting the fungi to an avirulent phenotype (De Witt *et al.*, 1993). The function of this protein is still not known.

I.6.3 Bacterial *hrp* Genes

A further level of compexity is added to the already complicated cultivar/race-specific interaction, by the isolation and characterization of a set of genes, from phytopathogenic bacteria, which are required for growth and pathogenicity *in planta* (not *in vitro*) and for the elicitation of the hypersensitive reaction. These genes were labelled *hrp* (for hypersensitive reaction and pathogenicity) because it was discovered that mutations in these genes resulted in partial or complete inability of the pathogen to cause disease in the host, coupled with the inability to cause HR in a non-host (Lindgren *et al.*, 1986).

The *hrp* genes are all chromosomally located and the majority of *hrp* genes (9-13) are clustered in a region of about 25 kbp, although in *E. amylovora* this cluster covers approximately 40 kbp (Genin *et al.*, 1993; Wei *et al.*, 1992; Xiao *et al.*, 1992). Two major groups of phytopathogenic bacterium appear to exist based on DNA cross-hybridization and/or on heterologous complementation of *hrp* genes. One group includes the *P. solanacearum* and *X. campestris* pathovars and the other includes the pathovars of *P. syringae* and *E. amylovora*. The transcriptional regulation of *hrp* genes has been elucidated to some degree in *Erwinia*, *Pseudomonas* and *Xanthomonas* species; *hrp* genes have also been cloned and sequenced. These studies have shown that *hrp* genes are repressed when bacteria are grown in rich medium, but are induced when grown in minimal medium in the presence of certain sugars or sugar alcohols; low pH and low osmolarity were also found to induce *hrp* genes (Rahme *et al.*, 1992). These results demonstrate that *hrp* genes are induced by a series of environmental conditions which correspond closely to the plant apoplast (low pH of approximately 5.5, low osmolarity and the presence of certain sugars). Accordingly, *hrp* genes which are required for growth and pathogenesis *in planta*, are induced by environmental stimuli which correspond closely to the environmental conditions found in plants. Not surprisingly, induction of *hrp* genes occurs when bacteria are inoculated into plant leaf tissue, however, in some cases, a specific plant signal may also be required for maximal *hrp* gene induction (Rahme *et al.*, 1992).

Recently the function of specific *hrp* genes has been assigned and the most significant discovery is that several *hrp* genes are homologous to pathogenicity determinants of animal pathogenic bacteria such as *Yersinia* and *Shigella* (Genin *et al.*, 1993). The fact that these virulence determinants are involved in the secretion of proteins required for pathogenesis suggests an analogous role in phytopathogenic bacteria. The *hrp* genes of *P. solanacearum* have been found to correspond to a variety of pathogenicity genes in mammalian pathogens: *hrp A* codes for a protein which is 34% identical over its entire length to the Ysc C protein of *Y. enterolitica*; *hrp E* codes for a protein which is 44% identical to the putative Spa 47 protein of *S. flexineri*; *hrp I* codes for a putative lipoprotein of 269 amino acids which is 35% identical over its entire length to Ysc J lipoprotein of *Y. enterolitica*; *hrp O* codes for a putative protein of 690 amino acids which is 43% identical to the Lcr D protein of *Y. pestis* and 32% identical over 189 amino acids to a partial open reading frame located 5' of the *spa 15* locus of *S. flexineri*; *hrp T* codes for a protein which is 40% identical to the putative protein encoded by the *spa 24* gene of *S. flexineri*. The structural genes for these proteins in the corresponding organisms have been shown to map on virulence plasmids and have been demonstrated to be (or are suspected of being) involved in the signal independent secretion of extracellular proteins (called YOPS in *Yersinia* and Ipas in *Shigella*). In both *Yersinia* sp. and *Shigella* sp., these proteins have been shown to be crucial for pathogenicity and host-cell invasion. DNA probes of *hrp A*, *hrp I* and *hrp O* hybridized to cosmid clones carrying the *hrp* gene clusters from of *X. campestris* pv. *campestris*, *P. syringae* pv. *phaseolicola* and *E. amylovora*, thus, the presence of homologous *hrp* genes in these organisms was confirmed (Genin *et al.*, 1993). Recently the *hrp I* gene from *P. syringae* pv. *syringae* 61 was sequenced and the putative protein was found to be highly homologous to Hrp O of *P. solanacearum* and Hrp C2 of *X. campestris* (Huang *et al.*, 1993). A common feature of all the preceding proteins is their involvement (or probable involvement) in the trans-membrane transit of macromolecules. Based on this information, it is hypothesized that *hrp* genes are involved in the secretion of macromolecules which are involved in pathogenesis and consequently, may also elicit the plant defense response. *Hrp* mutants retain the ability of the wild-type to secrete cellulolytic and pectinolytic enzymes, therefore, it is assumed that as in *Yersinia* and *Shigella*, the *hrp* gene secretion pathway of phytopathogenic bacteria might be specific for unknown pathogenicity proteins specifically involved in pathogenesis *in planta.*

In *P. syringae* pv. *phaseolicola* a cascade control of *hrp* gene expression is theorized (Miller *et al.*, 1993). Two loci designated *hrp RS* and *hrp L* are involved in the regulation of several *hrp* operons. The activation of the entire *hrp* locus during plant-pathogen interactions requires *hrp S*, which was found to encode a 34 kDa protein that shares sequence similarity to NtrC family of transcriptional regulator proteins. The Hrp R protein is 60% identical in amino acid sequence to the Hrp S protein. Both proteins bear homology to proteins involved in the two step regulatory system and possess a C terminal helix-turn-helix motif that may be involved in DNA binding. Both *hrp S* and *hrp R* undergo a dramatic induction at the infection site *in planta*; while *hrp S* was found to require some unidentified plant signal for induction and was induced in both the compatible and the incompatible interaction. The *hrp L* gene was shown to positively regulate the *hrp* locus, but it has not been characterized at the molecular level. The *hrp A*, *B*, *C* and *D* were all demonstrated to be dependent upon *hrp R*, as well as environmental factors such as pH and osmolarity, for expression. Four Hrp proteins from *P. syringae* pv. *phaseolicola* were predicted to be membrane-associated based on computer predictions and homology to other proteins. For example, the *hrp F* gene encodes a protein that resembles the Ysc J lipoprotein of *Y. enterolitica* and is, therefore, homologous to Hrp I of *P. solanacearum* .

Interestingly, it has been demonstrated that *avrRpt2* and *avrPto*, two avirulence genes from *P. syringae* pv. *tomato*, specifically require *hrp SR* and *hrp SL*, respectively, for transcription (Salmeron and Staskawicz, 1993; Innes *et al.*, 1993). The induction of *avrPto*, which is under the control of *hrp S* and *L*, occurs equally as well, in resistant and susceptible tomato cultivars, indicating that the *Pto* resistance gene does not control the expression of *avrPto* in tomato leaves and therfore, the transcriptional activation of *avrPto* is not at the molecular level the reason of race/cultivar specificity. Significantly, nine *P. syringae* avirulence sequences, including the two mentioned above, possess this consensus sequence in the promoter regions of their genes. This consensus region designated the "harp box", was also found upstream in the promoter region of four *P. syringae* pv. *phaseolicola hrp* operons which also require the *hrp SR* locus for expression. In addition, Huynh *et al.* (1989) have shown that *hrp* gene regulation is required for the expression of *avr B* in *P. syringae* pv. *glycinea*. In contrast, the avirulence gene *avr Bs3* from *X. campestris* was found to be induced, *in planta*, independently of the *hrp* locus; however, the avirulence function associated with the gene was only present in the presence of the *hrp* locus (Knoop *et al.*, 1991). Generally, bacterial avirulence genes can only be examined for avirulence activity in *hrp*-positive bacterial strains. The co-regulation of avirulence genes and the dependence for avirulence function on the *hrp* locus suggests that avirulence genes may be involved virulence and that avirulence gene products may interact with *hrp* gene products. This speculative association between *hrp* genes/gene products and *avr* genes/gene products, may modulate pathogenicity and dictate host-specificity at the molecular level. The most obvious theory is that *hrp* genes may be involved in the export of *avr* gene products; recent evidence demonstrates that proteinaceous necrogenic factors are in fact secreted by phytopathogenic bacteria *via* the *hrp* regulon.

A proteinaceous elicitor of the HR was isolated from *E. amylovora*, the bacterium that causes fire blight in pear, apple and other plants of the *Rosaceae* (Wei *et al.*, 1992). The elicitor named harpin is an acidic, heat-stable, 44 kDa protein that can elicit the HR in tobacco and several other plants. The *hrp N* gene which encodes harpin is located in the 40 kbp *hrp* gene cluster. Transposon mutagenized *hrp N* mutants did not elicit the HR in tobacco and were unable to cause disease symptoms on pear fruit. Mutations in several other *hrp* genes, other than *hrp N*, resulted in bacterial cells that could not elicit the HR unless first disrupted by sonication; this suggests that the products of these *hrp* genes are involved in the translocation of harpin to the cell surface.

A 34.7 kDa protein was isolated from *P. syringae* pv. *syringae* which was able to elicit the HR in tobacco plants (He *et al.*, 1993). The protein, designated harpin$_{Pss}$, is dissimilar in its overall amino acid sequence to harpin$_{Ea}$ (from *E. amylovora*). Low levels of amino acid sequence-similarity were detected, between harpin$_{Pss}$ and harpin$_{Ea}$, in a 22 amino acid stretch, however, the significance of this similarity remains unknown; this sequence similiarity could be involved in a secretion mechanism. Harpin$_{Pss}$ and Harpin$_{Ea}$ proteins showed no significant amno acid homology to deposited sequences in the databases. Both proteins are generally hydrophillic and possess no signal-sequence. Harpin$_{Pss}$ possesses two sets of amino acid sequences (11 amino acids long) which are directly repeating; both sets were found to be essential for elicitor activity. Using Southern and Western blotting analysis, homologs of the harpin$_{Pss}$ gene (*hrpZ*) and the harpin$_{Pss}$ protein were detected in *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *glycinea* race 4 and *P. syringae* pv. *tomato*. No homologous genes or proteins were detected in *X. campestris*; thus harpin$_{Pss}$ appears to be conserved only at the genus level. Harpin$_{Pss}$ was dependent upon *hrp H* for secretion. To determine if the HR induced by harpin$_{Pss}$ resulted from a direct toxic effect or from an active plant response leading to necrosis, the effect of various inhibitors of plant metabolism were noted on the harpin$_{Pss}$ necrotic action. In independent experiments, α-amanitin, cycloheximide, vanadate and lanthanum were each coinfiltrated with purified harpin$_{Pss}$ protein into tobacco leaf tissue. All four compounds prevented the HR elicitation by harpin$_{Pss}$, thus indicating that harpin$_{Pss}$ is not directly a toxic agent, but acts to signal plants to induce a dynamic defense response.

The role if any of harpins in determining host-specificity of *P. syringae* pv. *syringae* is still indeterminate and likely to be complex. Soybean leaves are insensitive to harpin$_{Pss}$, but they are resistant to *P. syringae* pv. *syringae* 61 and do elicit an HR when infiltrated with this bacterium. Weak necrosis was observed in bean leaves infiltrated with *P. flourescens* carrying the *hrpZ* gene. Harpin$_{Ea}$ is also ineffective in eliciting the HR in bean leaves, although bean is resistant to *E. amylovora*. Early indications demonstrate that the sensitivity to harpin$_{Pss}$ varies amongst plants, with no obvious correlation to host-range. The limited data available fail to support, but do not eliminate a role for harpins in determining host-range and race-cultivar specific resistance.

A proteinaceous HR inducing factor was also isolated from *P. solanacearum*. The 23 kDa protein bore no homology to harpin and the structural gene was located just outside the *hrp* gene cluster (Arlat *et al.*, 1994). This protein was not considered to be a harpin since it was not required for virulence in tomato or petunia and even though no purified avirulence protein has been able to elicit an HR, the necrotic activity and *hrp* gene dependant secretion of the protein suggested to the authors that it could be an avirulence gene.

I.6.4 Plant Resistance Genes

The gene-for-gene hypothesis also predicts the existence of plant resistance genes corresponding to microbial avirulence genes. There are several examples of avirulence genes being cloned providing strong evidence for the gene-for-gene hypothesis, however, almost nothing is known about the specific biochemical functions of corresponding plant resistance genes.

Specific disease resistance in plants often segregates in a Mendelian fashion, in a dominant or semidominant manner, suggesting either the presence of a single resistance gene, or the existence of several resistance genes in a tightly linked locus. The use of isogenic, or near isogenic lines introduced by Loegering and Harmon (1969), made it possible to study cultivar-specific resistance/susceptibility, with respect to single loci. There are at least 113 cases of plant/pathogen interactions which operate at the cultivar/race specific level. For example, there are at least five resistance genes in pea and bean postulated to recognize some of the known races of *P. syringae* pv. *pisi* and *P. syringae* pv. *phaseolicola* (Dangl *et al.*, 1992). In flax, 29 known genes that confer resistance to rust disease occur at loci designated K, L, M, R and P, of which, there are 1, 13, 7, 3 and 5, alleles or genes, respectively. In barley, a "single resistance specificity" against powdery mildew occurs at each of the following loc, *Mlg*, *Mlk* and *Mlp*, but 12 alleles are known at the *Mla* locus (Pryor, 1987). In soybean, the resistance genes *Rpg1*, *Rpg2*, *Rpg3* and *RPg4* have been identified to confer specific resistance to *P. syringae* pv. *glycinea* carrying the avirulence genes *avrA*, *B*, *C* and *D*; some of these resistance genes appear to be linked (Keen and Buzzell, 1991).

In one example in maize, a high rate of induced mutations was observed at the rust disease resistance locus *Rp1*. Studies of the *Rp1* locus indicated that 8 of the 9 alleles tested spontaneously generated susceptible mutants at frequencies of $1.5\text{x}10^{-4}$ for *Rp1d* to $7\text{x}10^{-3}$ for *Rp1g*, which is substantially higher than spontaneous mutation rates (Pryor, 1987). The high rate of mutation in plant resistance genes has lead to the hypothesis that a systematic mechanism may be involved in the formation of mutations. Similarly, avirulence specificity is readily mutable and the high rate of mutations in plant resistance loci correlates with mutation rates of avirulence in microbes (Gabriel and Rolfe, 1990). For example, *X. campestris* pv. *vesicatoria* carrying *avr Bs1* acquired virulence at a noticeably high frequency calculated to be $4\text{x}10^{-4}$ (Dahlbeck and Stall, 1979). Thus a hypothesized mutational mechanism in plants, could possibly be a system for the generation of new resistance phenotypes, to counteract the high rates of avirulence gene mutation and overall high genetic drift observed in microbes.

In *Arabidopsis*, two resistance genes, *RPM1* and *RPS2*, which confer resistance against *P. syringae* strains expressing the avirulence genes, *avrRpm1* (from pathovar *maculicola*) and *avrRpt2* (from pathovar *tomato*) have been mapped to regions of chromosomal DNA, with respect to RFLP and PCR markers. The *RPS2* locus has been mapped to chromosome IV (Yu *et al.*, 1993) and *RPM1* has been mapped to chromosome III (Debener *et al.*, 1991). In addition, the *RPM3* resistance locus conferring resistance to *P. syringae* isolates carrying *avrB* (an avirulence gene from *P. syringae* pv. *glycinea* which is homologous to *avrRpm1*) has also been mapped and was found to be tightly linked to the *RPM1* locus; thus the two loci may represent the same gene (Innes *et al.*, 1993). A yeast artificial chromosome (YAC) clone of 270 kbp that contains *RPM1* has been isolated, thus the cloning and sequencing of the plant resistance locus *RPM1* and/or *RPM3* is imminent.

Since *avrRpt2* also converts virulent *P. syringae* strains to avirulence on bean and soybean it is highly probable that these plants also contain *RPM2*, or a functionally homologous gene, presenting the theory that specific resistance genes may be conserved amongst different plant species. As discussed previously, the *avrRxv* gene from *X. campestris* pv. *vesicatoria* converts *Xanthomonas* pathogens of soybean, alfalfa, cotton and maize, to avirulence on their respective hosts (Whalen *et al.*, 1988). Thus it has been postulated that a common resistance gene, which is specific to *avrRxv*, may be conserved amongst these diverse plant species. At least five other avirulence genes from *X. campestris* and *P. syringae* are now known to confer avirulence on multiple plant species (Dangl *et al.*, 1992; Fillingham *et al.*, 1992; Innes *et al.*, 1993; Ronald *et al.*, 1992; Swarup *et al.*, 1992; Whalen *et al.*, 1991). The most elementary explanation of these observations is that plant resistance genes may have been conserved during evolution and speciation and are therefore common to several different plant species. This appears to be the case since functional homologs of the *Arabidopsis RPM1* exist in bean and pea (Dangl *et al.*, 1992).

Also of significance are the findings of Kiedrowski *et al.* (1992), who were the first to demonstrate a direct link between the rapid activation of an *Arabidopsis* defense gene (*ELI3*) and its dependence upon the *Arabidopsis RPM1* disease resistance locus. Only resistant *Arabidopsis* ecotypes possessing the *RPM1* locus accumulated high levels of the defense gene mRNA 5-10 hrs after inoculation with *P. syringae* carrying *avrRpm1*. Susceptible *Arabidopsis* ecotypes that did not possess *RPM1* accumulated the defense gene transcript only 15 hrs after inoculation.

Numerous different approaches have been suggested to clone plant resistance genes; these involve chromosome walking from linked marker genes or other markers, tagging with a transposable element, isolation of protein product by the use of affinity labelling with a labelled specific elicitor and finally complementation cloning (Ellis *et al.*, 1988).

At present two plant resistance genes have been cloned. In one case the mechanism of resistance is straightforward, while the other case is more ambiguous, although the signal transduction mechanism is specifically implicated has the mechanism of plant-resistance. *Cochliobolus carbonum* race 1 was discovered to be pathogenic only on certain inbred lines of maize. The presence of the Hc toxin (a cyclic tetrapeptide) permitted the fungus to infect certain highly inbred corn lines while standard breeding lines were resistant; this resistance trait behaved as a dominant, single, Mendelian locus when crosses were made between a susceptible and a resistant corn line and was designated the *Hm1* locus. The *Hm1* locus was targeted through transposon mutagenesis then by screening the mutagenized progeny for sensitivity to the fungus. Eventually the *Hm1* locus was cloned and sequenced and was found to contain a gene coding for an NADPH-dependent Hc-toxin-reductase. The Hc-toxin-reductase gene which inactivates the fungal toxin had apparently been lost in the inbred line thus this mechanism for resistance is quite straightforward (Johal and Briggs, 1992).

Of greater complexity is the *Pto* resistance gene from tomato. The *Pto* gene confers resistance to bacterial speck diseases caused by *P. syringae* pv. *tomato* possessing the *avrPto* gene. In tomato, over 27 loci have been identified that confer resistance to many agriculturally important fungi, nematodes and bacteria. Many of these loci, including *Pto*, have been mapped to small intervals on genetic linkage maps. Consequently, high density RFLP maps and a YAC library have allowed the map-based cloning of *Pto* from tomato (Martin *et al.*, 1993). A tomato *Pto* cDNA clone was isolated and sequenced and found to encode for a 321 amino acid hydrophillic protein.

Comparison of the putative resistance protein with protein sequences in the databases revealed homology with the catalyst domains of many plant, mammalian and lower eukaryotic, serine-threonine protein-kinases. Eleven subdomains, including 15 invariant amino acids characteristic of protein-kinases, were present in the expected locations. When the cDNA clone was transformed into isogenic tomato plants that were sensitive to *P. syringae* pv. *tomato*, the plants became resistant, thus proving that the putative clone was in fact the *Pto* gene and that it could confer resistance to closely related tomato plants lacking the resistant *Pto* allele. This was the first experiment demonstrating the cloning of a plant resistance gene and provides from the plant side, convincing proof for the gene-for-gene hypothesis.

The presence of short DNA segments homologous to *Pto* in proximity to the *Pto* gene suggested the strong likelihood of recombination events in the region which may have occurred, possibly shifting domains of gene family members and generating new specificities. Such intralocus recombination events would be increased by clustering and could be a mechanism of generating unique resistance specificities to phytopathogenic microbes. Genomic DNA from several monocotyledonous and dicotyledonous plants was probed with a *Pto* gene probe and the presence of homologous sequences in all the tested plants was verified. Some plants showed multiple banding indicative of a gene family, thus, the *Pto* resistance gene is conserved in a variety of plants.

The Pto protein possesses no obvious membrane spanning or extracellular domain that would suggest a function as an external receptor for a pathogen signal. At the NH_2 terminus a potential myristillation site occurs, suggesting that Pto might be membrane associated in a manner similar to that of the myristolated tyrosine protein-kinase $p60^{src}$. Association with the cytoplasmic membrane could place Pto in proximity to an extracellular receptor having a cytoplasmic domain. A widely accepted model based on the genetics of the gene-for-gene interactions, on the rapid depolarization of the plant cell membrane and rapid intracellular flux of calcium ions observed in resistant plant cells, suggests that a plant resistance gene product acts as a cell-surface receptor for the products (direct or indirect) of microbial avirulence genes (Gabriel *et al.*, 1988). A given interaction with the proper avirulence gene-specified-ligand triggers intracellular signaling events, activating *de novo* plant defense gene expression, culminating in a resistance response (Gabriel *et al.*, 1988; Lamb *et al.*, 1989). Based on this theory, a common signal transduction pathway could then transfer an array of separate signals from the exterior of the plant cell, by means of a family of distinct receptors, to a set of common cytoplasmic target molecules (possibly transcription factors), which then induce *de novo* host-defense gene expression and culminate in plant resistance.

I.7 *ARABIDOPSIS*, A MODEL SYSTEM FOR PLANT-PATHOGEN STUDIES

Both partners of a gene-for-gene interaction, the *avrPto* and the *Pto* genes, have been cloned, but this has provided only clues as to how the interaction works on the molecular level. This perplexing interaction will only be solved by the discovery of further molecular components of the interaction in other plant systems, which will provide more examples of how plant resistance functions universally in plants. There is little known about what happens in plant cells, between the time a plant is exposed to an incompatible microbe and the time at which new defense mRNA's appear. Biochemical investigations of intracellular signalling, following pathogen recognition, have in the past depended largely upon on the use of plant cell suspension systems in which the transcriptional activation of defense related genes was triggered by non-race-specific fungal elicitors (Douglas *et al.*, 1992). In recent years, the small cruciferous plant *Arabidopsis thaliana* has become the model plant for studying plant molecular genetics. *Arabidopsis* offers several advantages over other plants which make it a desirable plant for studying plant molecular genetics. *Arabidopsis* has a short generation time, a large seed set, a small genome and is susceptible to *Agrobacterium*-mediated transformation (Meyerowitz, 1989). Its relatively small genome has allowed development of high resolution RFLP maps and YAC libraries. Recently, *Arabidopsis* has also been established as a model host in which to study the molecular genetics of the plant defense response (Davis, 1992). As discussed previously, *P. syringae* strains that are pathogenic and nonpathogenic in *Arabidopsis* have been identified and several bacterial avirulence genes (*avrRpt2*, *avrB* and *avrRpm1*) have been cloned. Reports of specific and rapid preferential induction of defense genes (PAL and *ELI3*), during the incompatible interaction between *Arabidopsis* and *P. syringae* strains carrying an avirulence gene, indicate that a communication occurs between the plant and the bacteria in a short interval after exposure to the avirulent microbe (Dong *et al.*, 1991; Kiedrowski *et al.*, 1992). Thus, there is specific mechanism of "communication" between the avirulence gene (i.e., *avrRpt2*) and the complementary resistance gene (i.e., *RPT2*), which leads to PAL gene induction and HR resistance. The nature of the interaction is yet to be determined.

I.8 THESIS OBJECTIVE

The luciferases (i.e., bacterial and firefly), which catalyze light emission, are the most versatile class of noninvasive reporter genes available and thus offer a unique potential as an *in vivo* reporter gene system. Specifically, the *lux F* reporter gene developed from the marine bacterium *Vibrio harveyi* has been recognized in its distinct capacity as an *in vivo* reporter gene system in plants (Langridge *et al.*, 1989; Koncz *et al.*, 1990; Jiang *et al.*, 1992). In *V. harveyi*, the luciferase enzyme exists as a stable heterodimeric oxidase composed of the Lux A and the Lux B subunits. This bacterial luciferase catalyzes the oxidation of reduced flavin mononucleotide and long chain aldehydes (e.g., *n*-decanal) which generates blue-green light at a wavelength of 490 nm. The overall reaction is shown below:

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + 0.1\ h\nu\ 490\ \text{nm}$$

The structural genes *lux A* and *lux B* have been fused into a single open reading frame, *lux F*, encoding a monomeric luciferase with a specific activity and emission spectrum similar to the wild-type enzyme (Escher *et al.*, 1988).

The goal and main topic of this thesis is to create an *in vivo* model system for monitoring plant-pathogen interactions between *Arabidopsis* and phytopathogens. By creating a transcriptional fusion between the *Arabidopsis PAL1* promoter (Ohl *et al.*, 1990) and the *lux F* reporter gene, one could monitor *PAL1* promoter induction *in vivo*. Very little is known about the mechanism involved in the activation of this defense gene and we remain largely ignorant of the many facets of signalling in plants. A model system for studying *PAL1* promoter induction could help to elucidate the mechanism whereby a potential pathogen is perceived and specific steps in the signal transduction which relate the presence of a pathogen to the genome. By mutagenizing plants that contain the *PAL1* promoter-*lux F* gene fusion, and by screening the progeny of mutagenized plants for aberrant luciferase expression, both "*PAL1*-constitutive" and "*PAL1*-noninducible" mutants could be isolated. Progeny of this second type of mutant would presumably be mutagenized in a resistance gene encoding a component of the signal transduction pathway, imperative for the transcriptional activation of defense genes. A similar system has been suggested for use in the isolation of novel elicitors because it could provide a rapid assaying tool for screening for elicitor activity

(Doerner *et al.*, 1990). Such a system could also provide new information on the temporal or spatial nature of *PAL1* promoter regulation in plants. In this thesis I will describe the development of a model *in vivo* system to monitor *PAL1* promoter regulation in *Arabidopsis* plants, in response to microbial infection.

II. MATERIALS AND METHODS

II.1 ENZYMES, REAGENTS AND SUPPLIES

Restriction endonucleases and the Klenow fragment of *E. coli* DNA polymerase where obtained from Boehringer Mannheim and T4 DNA ligase was obtained from Boehringer Mannheim and from Gibco BRL; all were used in accordance with the manufacturer's specifications with the 5 × or 10 × reaction buffers included, unless otherwise stated. *Taq* polymerase was purchased from Perkin-Elmer Cetus and Gibco BRL and was used in conjunction with the included concentrated buffer stocks, or with a standard laboratory-prepared *Taq* buffer (see II.8.1). Sequenase®* and Sequenase®* Version 2.0 sequencing kit were purchased from United States Biochemicals (USB). Exonuclease III and S1 nuclease were purchased from Gibco BRL and Boehringer Mannheim, respectively and were used with buffers recommended by Sambrook *et al.* (1989).

[α^{32}P]-dATP and [α^{35}S]-dATP were purchased from Amersham Corporation. The dNTP's were obtained from Boehringer Mannheim, as 10 Molar stocks and these were diluted down to working-stocks as needed for PCR amplification, PCR sequencing reactions and random primer labelling. The ddNTP's for PCR Cycle sequencing were obtained as concentrated 10 mM stocks from Boehringer Mannheim, or from Pharmacia and were used to make the various termination mixes for automated DNA sequencing.

Oligonucleotide primer for PCR generation of the *PAL1* promoter were provided by K. Roy (Department of Microbiology, University of Alberta). The *lux A* primers were synthesized on an ABI 380B DNA synthesizer (Plant Molecular Genetics, University of Alberta). The fluorescent dye-labelled primers (-21m13 Universal and Reverse) used for automated DNA sequencing were purchased from ABI.

Bacterial media used in this study was purchased from Difco® and Sigma®

chemical companies. MS basal salts medium (Murashige and Skoog, 1962) was purchased from Sigma® Chemical Co. in the form of pre-weighed, premixed, "ready-to-dissolve" powder (1 bottle of MS salts = 4.2 gm); the organic compounds were added upon dissolving of the MS salts in 1L ddH_2O. A concentrated MS vitamin stock was prepared and stored frozen at -20° C; it was melted and added to MS medium before autoclaving as required.

Cefotaxime (Claforan®) was purchased from Roussel Canada Inc. and was supplied in the form of a sterile hygroscopic powder; all other antibiotics were purchased from Sigma® Chemical Company. All the chemicals and reagents used in this study were of reagent grade.

II.2 PLASMIDS, BACTERIAL STRAINS, CULTURE CONDITIONS AND PLANT MATERIALS

The plasmids used in this study were obtained from the following sources; pBluescript KS+ (Stratagene Cloning Systems) was obtained from L. Bögre (Plant Molecular Genetics, University of Alberta), pPCV701*luxFM1* (Jiang *et al.*, 1993) was obtained from C. Jiang (Plant Molecular Genetics, University of Alberta). The plasmids pPAL45 and pGL101 are the subjects of this study.

The various strains of bacteria used in this study were obtained from the following sources; *Escherichia coli* DH5α (*supE*44Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi*1 *relA*1) (Bethesda Research Laboratories) was obtained from R. Mayerhoffer (Plant Molecular Genetics, University of Alberta) and *E. coli* S-17 (F^- *pro* *recA*1 $r_k^-m_k^+$, RP4-2 integrated, Tc::Mu Km::Tn7 Sm^r Tp^r) (Simon *et al.*, 1983) was kindly provided by C. Jiang. *A. tumefaciens* GV3101 (pMP90RK) (Koncz and Schell, 1986) was obtained from L. Bögre. *P. syringae*, pathovars *tomato* JL1065 (nalidixic $acid^r$) and *maculicola* ES4326 ($streptomycin^r$) (Dong *et al.*, 1991), were obtained from F.

Ausubel (Department of Molecular Biology, Massachusetts General Hospital).

E. coli strains were maintained and grown on LB agar medium (Sambrook *et al.*, 1989); *E. coli* strains harbouring pBluescript KS+, pPAL45 (and deletion derivatives of pPAL45) or pGL101 were grown on LB medium supplemented with 100 µg/mL ampicillin. Competent *E. coli* DH5α and S-17 cells were prepared as described by Inoue *et al.* (1990) and were stored in 100-300 µL aliquots at -70° C until required.

A. tumefaciens cells were maintained on YEB agar medium (Sambrook *et al.*, 1989) supplemented with gentamycin (25 µg/mL), kanamycin (25 µg/mL) and rifamycin (100 µg/mL). *A. tumefaciens* cells carrying pGL101 were maintained on the same medium with additional kanamycin (50 µg/mL instead of 25 µg/mL) and 100 µg/mL carbenicillin for plasmid maintenance.

P. syringae, pathovars *tomato* and *maculicola*, were maintained on King's medium B (King *et al.*, 1954) supplemented with nalidixic acid (15 µg/mL) and streptomycin (150 µg/mL), respectively. *Pseudomonas* cultures were re-streaked on fresh medium every 3-4 weeks for 4 passages (3-4 months) and then fresh plate cultures were reestablished from glycerol stocks.

Glycerol stocks of all the bacterial strains were prepared by growing a 2 mL liquid culture in the appropriate medium, supplemented with antibiotics if required for plasmid maintenance, to early stationary phase. Autoclaved glycerol (150 µL) was added to 850 µL of the forementioned bacterial culture. The mixture was vortexed, frozen immediately in liquid nitrogen and stored at -70° C. When needed, small frozen chunks from the -70° C glycerol stock were scraped-off onto the appropriate agar medium using a sterile spatula or sterile forceps.

Seeds of *Arabidopsis thaliana* ecotypes *Landsberg erecta* and RLD were provided by B. Ayre (Plant Molecular Genetics, University of Alberta). Seeds were surface-sterilized by gently shaking in 1 mL of sterilization solution (0.525% sodium hypochlorite, 0.02% Triton X-100), for 30-45 mins in a 1.5 mL Eppendorf tube. The seeds were briefly allowed to sediment to the bottom of the tube, the sterilization solution was drawn-off and the seeds were washed twice (2 mins each) in sterile ddH$_2$O. The sterile seeds were re-suspended in 200-500 µL of sterile ddH$_2$O and were pipetted onto sterile MS medium supplemented with 1.25% [w/v] sucrose and solidified with 0.22% [w/v] gellan gum (Gel-rite™, Adams Scientific), hereafter referred to as MS 1.25%.

Non-transformed *Arabidopsis* plants were grown on sterile MS 1.25% unless stated otherwise. Transformed plants were always grown on this identical MS 1.25% medium supplemented with 65 µg/mL kanamycin, as a selection against nontransformed plants and selection for the presence of functioning T-DNA insertions.

Transformed *Arabidopsis* plants were grown for 2-3 weeks on selection medium and were then harvested for DNA isolation. For the studies of microbial-induced luciferase activity *in planta*, transformants were grown for 1½-2 weeks on MS 1.25% selection medium, then the kanamycin-resistant plantlets were transferred to small square plastic pots (2½" square × 2⅓" deep) containing Metro-Mix 290 potting mix (Terra-Lite Inc.) and were grown in the greenhouse at 24° C under a 12 hr photoperiod (333 µE /m^2 sec) for 4-5 weeks. Plants were watered regularly every two days with regular tap water; no fertilization was required for healthy plant growth.

II.3 ISOLATION OF PLASMID DNA

Plasmid DNA was routinely isolated for restriction enzyme analysis when screening for a particular construct from *E. coli* DH5α using the minipreparation alkaline-lysis procedure described in Sambrook *et al.* (1989). For DNA sequencing using the Sequenase®* sequencing kit, the same procedure for isolating plasmid DNA template was followed, with the addition of a phenol and a phenol/chloroform (1:1) extraction, where only the phenol/chloroform extraction is indicated; in some cases the plasmid DNA used for sequencing, in addition to being extracted with phenol, was also further purified using Geneclean® (Bio. 101 Inc.). For the isolation of plasmid DNA from *A. tumefaciens* GV3101, cells were pelleted first and were then re-suspended in 1 M NaCl as a "wash step"; cells were then pelleted, the NaCl solution was drawn-off and the same procedure cited above was employed.

Large-scale purification of plasmid DNA was also accomplished by equilibrium-centrifugation in a CsCl-EtBr gradient as outlined in Sambrook *et al.* (1989), with one minor modification, the first centrifugation step to remove protein-EtBr complexes was omitted. The crude plasmid preparation was directly centrifuged for 18 hrs at 20° C in an Ultracentrifuge (Beckmann L8-m) at 55 000 rpm using a vti 65.2 class H rotor.

II.4 AGAROSE GEL ELECTROPHORESIS

Agarose gels (0.8-1.0%) of various sizes were routinely used to: i) analyze for desired constructs based on restriction endonuclease digestion, ii) isolate DNA fragments (inserts or vectors) used in plasmid construction and iii) isolate templates for making radiolabelled probes for plant genomic DNA hybridization. The desired amount of agarose powder was dissolved in 0.5 × TBE by boiling. Upon cooling, EtBr was added to a final concentration of 0.5 μg/mL. The liquid agarose was mixed briefly and was poured into the desired gel mould. TAE agarose gels (1%, 16 cm × 16 cm) were used for separating restriction endonuclease digests of total plant genomic DNA and were prepared in the same way as the TBE agarose gels.

II.5 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS

DNA fragments used in cloning and as templates for radiolabelled probes were routinely isolated using Geneclean® or by electroelution. Geneclean® was used according to the manufacturer's specifications; it often gave inconsistent yields. Therefore, electroelution was the favoured method and consistently furnished higher yields. The block of agarose, containing the desired DNA fragment, was cut from an agarose gel and was placed in a small segment of dialysis tubing (2 cm. diameter) which was prepared according to procedure described in Sambrook *et al.* (1989). The tubing was filled with electrophoresis running buffer, clipped shut and placed in the electrophoresis tank. A voltage of 100-150 V was applied for approximately 5-10 mins and the fragment was examined under UV-light to determine whether any DNA remained in the agarose block. The 300 µL of 0.5 × TBE containing the DNA was collected, extracted with phenol and the DNA was precipitated with 30 µL of 3 M NaOAc (pH 5.2) and 2 volumes of ice-cold ethanol (95%). The DNA was pelleted, washed with 75% ethanol, dried and dissolved in ddH_2O to a final concentration of 35-100 ng/µL.

II.6 QUANTIFICATION OF DNA

Plasmid DNA was quantified according to Sambrook *et al.* (1989), where 1 A_{260} unit of plasmid DNA is equivalent to 50 µg plasmid/mL. The ratio between absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of the nucleic acid preparation. Pure preparations of DNA have A_{260}/A_{280} values of 1.8. With protein or phenol contamination, the A_{260}/A_{280} value will be significantly less than 1.8 and accurate quantification of the amount of nucleic acid will not be possible.

The concentration of *PAL1* and *lux A* oligonucleotide PCR primers were determined using absorbance readings at 260 nm. The extinction coefficient at 260 nm calculated for each specific primer. For any given oligonucleotide the number of times each base appears in the primer is multiplied by its extinction coefficient; the resulting 4 values are added to obtain the extinction coefficient (ϵ) at 260 nm for the entire oligonucleotide (Sambrook *et al.*, 1989). The extinction coefficients (ϵ) of each nucleotide base at 260 nm are: dGTP= 11.7 mL/μmol, dCTP=7.3 mL/μmol, dATP=15.4mL/μmol and dTTP=8.8mL/μmol.

II.7 ISOLATION OF PLANT GENOMIC DNA FOR PCR

Isolation of *Arabidopsis* DNA for PCR amplification of the *PAL1* promoter was essentially the same as Doyle and Doyle (1990). *Arabidopsis thaliana* plants (ecotype *Landsberg erecta*) were grown in Metro-Mix 290 in the greenhouse for 3-4 weeks and 2-3 grams of plant leaves were used as a source of plant tissue. The purified DNA was quantified by running a known amount of λ DNA and plant DNA on a TBE agarose mini-gel, since the quantification by absorbance at 260 nm generally gave unreliable results. Plant DNA was stored at -20° C.

II.8 CLONING OF THE *ARABIDOPSIS PAL1* PROMOTER

II.8.1 Polymerase Chain Reaction

Two primers designed for the generation of the full-length 1.8 kbp *A. thaliana* *PAL1* promoter which would incorporate unique *Sal*I and *Bam*HI restriction enzyme sites at the termini of the final product:

(primer #1- 5'-*TAGGGAT*CCTGCTAGTCTGCTACGTGGAA-3')
*Bam*HI

(primer #2- 5'-*TAGGTCG*ACTAAGAGGAGATTACAAGATCTC-3')
*Sal*I

The annealing temperatures of 70° C and 66° C for primer #1 and #2, respectively, were calculated using the formula $T_a = 4^\circ C (G+C) + 2^\circ C (A+T)$ (Hamill *et al.*, 1991); where G+C represents the number of guanine and cytosine residues and A+T represents the number of adenine and thymine residues in the primer. The "extra" nucleotides at the 5' end of each of the oligonucleotide primers (indicated in italics), designed to create restriction endonuclease sites on the termini of the full-length PCR product, did not have complementary bases in the genomic template and were not included in the annealing temperature calculations.

Genomic DNA (200 ng) and 50 pmol of each primer were used in a 50 µL PCR reaction mixture containing a final concentration of 10 mM Tris-HCl, (pH 8.3 at 25° C), 1.5 mM $MgCl_2$, 50 mM KCl (from a 10 × *Taq* reaction buffer prepared in the lab) and 0.2 mM of each dNTP. The PCR reaction mixture was overlaid with approximately 50 µL of mineral oil and was incubated in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 30 cycles of 3 mins at 92° C, 2 mins at 61° C and 3 mins at 72° C; followed at the completion by a single 10 min 70° C extension cycle. A slightly lower annealing temperature was used in the PCR amplification since the ones calculated above appeared higher than required. The mineral oil was removed by first freezing the samples at -20° C for 1-2 hrs and then withdrawing the unfrozen top layer of mineral oil by vacuum aspiration.

II.8.2 Vector preparations for cloning

CsCl-purified pBluescript KS+ DNA (1 µg) was digested consecutively with *Bam*HI and *Sal*I. The PCR product (1 µg) was electrophoresed through an agarose gel, isolated from the gel with Geneclean® and digested with *Bam*HI and *Sal*I. Following restriction enzyme digestion, both the vector DNA and the PCR fragment were purified by electrophoresis on an agarose gel and isolated using the Geneclean® procedure.

II.8.3 Ligation of DNA and transformation into *E. coli*

The *Sal*I and *Bam*HI digested vector (60-80 ng) and 300 ng of the PCR product were ligated with 1 Unit of T4 ligase in a 10 μL reaction volume. The ligation reaction was incubated at room temperature for 5 hrs and 5 μL of the ligation mixture was then used to transform 100 μL of competent *E. coli* DH5α according to the heat-shock method (Sambrook *et al.*, 1989); the remaining 5 μL was stored at -20° C. The transformed bacteria were plated-out in 2 portions (10% and 90% aliquots) onto 2 LB-ampicillin (100 μg/mL) agar plates. "Blue/white colour selection" (Sambrook *et al.*, 1989) was initially used to screen for transformants possibly carrying the desired plasmid insert; independent transformants were then examined by restriction enzyme analysis of miniprep. isolated plasmid DNA. An *E. coli* colony was identified which carried the pBluescript KS+ plasmid which contained the predicted *Sal*I-*Bam*HI 1.8 kbp insert; this plasmid was designated pPAL45.

II.9 DNA SEQUENCE ANALYSIS

Initially the nucleotide sequence of the terminal ends of the 1.8 kbp cloned PCR fragment were determined using the chain termination DNA sequencing method (Sanger *et al.*, 1977). Sequenase®* Version 2.0 sequencing kit was used to sequence 3-5 μg of double-stranded, CsCl-purified, plasmid pPAL45; [α^{35}S]-dATP was used as the radiolabel and the -21m13 Universal and Reverse primers included in the Sequenase®* sequencing kit were employed to verify the identity of the cloned PCR fragment. Double-stranded plasmid DNA template was denatured in 0.2 M NaOH, 0.2 mM EDTA for 4 mins and was precipitated with 3 M NaOAc (pH 5.2) and ice-cold ethanol (95%). Sequencing protocols were carried out as specified by the manufacturer, USB. The products of the sequencing reactions were separated on a 0.20 mm thick, 6% denaturing polyacrylamide gel (approximately 43 cm × 38 cm), using 1 × TBE and an International Biotechnologies Inc. sequencing apparatus (Model STS-45). A voltage of approximately

1800 V was applied for 5-6 hrs. After 6 hrs, the gel was removed onto a piece of gel-sized Whatmann® 3 mm chromatography paper, covered with cellophane and dried in a gel-dryer (Slab Gel Dryer Model SE1160, Hoeffer Scientific Instruments). The dried gel was autoradiographed using Kodak X-OMAT™ X-K1 X-ray film for 24-48 hrs.

Subsequently, a series of nested deletions were generated into the *PAL1* promoter (in plasmid pPAL45), by unidirectional Exo III/S1 nuclease digestion, according to the method of Sambrook *et al.* (1989). The plasmid pPAL45 was digested with *Sac*II and *Bam*HI to produce a 3' protruding, exonuclease-protected terminus on the vector side and an adjacent 5' protruding, exonuclease-sensitive terminus at the initiation of the insert. The linearized plasmid was then treated with Exo III and S1 nucleases. The nuclease-treated plasmid was then ligated with T4 ligase (Sambrook *et al.*, 1989) and the ligated plasmid was transformed into competent *E. coli* DH5α cells. Plasmid DNA was isolated by the miniprep. method (II.3) from individual ampicillin-resistant *E. coli* colonies. The isolated plasmids were screened for differences in size of the *PAL1* promoter insert by restriction enzyme analysis. Clones were identified which appeared to give an overlapping series of unidirectional, 200-300 bp, nested deletions. The DNA sequencing of the *PAL1* promoter insert was continued using Sequenase®* sequencing kit (USB) and the -21 m13 Universal primer as described above. The same clones were sequenced using an ABI (Applied Biosystems Inc.) model 370A DNA sequencer equipped with an Apple™ Macintosh IIc computer and ABI Cycle sequencing software. Double-stranded miniprep. plasmid template was isolated as specified in the ABI *TAQ* Dyedeoxy™ Terminator Cycle Sequencing Kit Manual (1992); specifically, a Modified Mini-Alkaline Lysis/PEG precipitate procedure was employed to grow bacteria and isolate suitably "clean" plasmid DNA template. The *Taq* cycle sequencing method using -21m13 Universal dye-labelled primers and ddNTP terminator mixes was followed as outlined in the ABI manual; Gibco BRL *Taq* polymerase was employed with the included concentrated buffer stock for all the sequencing reactions. Washing and cleaning of ABI glass plates, casting of gels, polyacrylamide reagents, running buffer preparation and the general electrophoresis procedures were all performed as specified in the ABI 370A DNA sequencer user manual.

II.10 PREPARATION FOR *AGROBACTERIUM*-MEDIATED PLANT TRANSFORMATION

II.10.1 Cloning of the *PAL1* promoter into the plant expression vector pPCV701*luxFM1*

The vector pPCV701*luxFM1* was sequentially digested with *Bam*HI and *Sal*I and the products were separated on a TBE agarose gel; the slow migrating vector (approximately 12 kbp) was electroeluted and treated as in II.5. In a similar way, the plasmid pPAL45 was also digested with *Bam*HI and *Sal*I and the *PAL1* promoter fragment was isolated using electroelution. The vector DNA (180 ng) and 40 ng of insert DNA, which equals a 1:1.5 vector to insert ratio, were used in a 10 µL total reaction volume. Ligation of the *PAL1* promoter into pPCV701*luxFM1* was carried out at 15° C for 6 hrs using 1 Unit of T4 ligase. After ligation, 1/2 of the ligation mixture was used to transform 100 µL of competent *E. coli* DH5α cells and the ligation mixture remaining was frozen at -20° C. Ampicillin-resistant *E. coli* colonies were analyzed by miniprep. isolation of plasmid DNA followed by restriction enzyme mapping. A plasmid with the correct insert was isolated; this novel construct was designated pGL101 (*PAL1-lux F*).

II.10.2 Conjugal transfer of pGL101 into *Agrobacterium*

Competent *E. coli* S-17 cells were transformed with pGL101 by the heat-shock method cited previously. Transformed *E. coli* cells were grown overnight on selection medium and individual colonies were analyzed using restriction enzyme mapping of plasmid DNA to verify the presence of pGL101.

The donor cells, *E. coli* S-17 cells harbouring pGL101, were grown overnight at 37° C on YEB supplemented with Mg^{2+} (2 mM) and ampicillin (100 μg/mL). The recipient cells, *A. tumefaciens* GV3101, were grown overnight at 28° C on YEB supplemented with Mg^{2+}(2 mM) gentamycin (25 μg/mL), kanamycin (25 μg/mL) and rifamycin (100 μg/mL). The donor bacteria and the recipient bacteria were each separately scraped-off their agar medium into separate sterile tubes using an inoculating loop and were re-suspended in 5 mL of YEB liquid medium supplemented with Mg^{2+}(2 mM). The cells were pelleted and the liquid medium was drawn-off. The cells were re-suspended in 1 mL each of YEB with Mg^{2+} and the $O.D._{600}$ of the two suspensions were measured. The cell suspensions were either diluted or concentrated by pelleting and re-suspending, so that in a 50-200 μL aliquots of the donor cell suspension, approximately 6×10^8 cells were present, and in a 50-200 μL of the recipient cell suspension, 2×10^8 cells were present. The cells were plated-out (6×10^8 donor cells and 2×10^8 recipient cells) and mixed by spreading onto a YEB (Mg^{2+}) plate. This plate was incubated at 29° C. After 24 hrs growth a few loops of bacteria were scraped-off and re-suspended in 10 mM $MgCl_2$. This suspension was briefly washed and re-suspended in the same solution. The concentration of the bacterial suspension was determined by measuring the $O.D._{600}$ and three different amounts of cells (5×10^7, 1×10^8 and 4×10^8) were plated on YEB (Mg^{2+}) agar medium supplemented with Gm^{25}, Km^{50}, Rif^{100} and $Carb^{100}$. Small, visible, pin-point colonies were growing on the surface of the medium after 24 hrs incubation at 28° C; after 36 hrs, isolated colonies were observed. The isolated colonies were re-streaked 3-4 times and grown at 28° C on the same YEB full-selection medium to isolate transconjugant *A. tumefaciens* colonies, and to insure no contamination by either *E. coli* donor cells or nontransformed *A. tumefaciens* cells.

Single colonies of transconjugant *A. tumefaciens* were examined, *in vivo*, for the presence of pGL101 by measuring luminescence in the low-light imager (Argus-100 Low-Light Imager, Hamamatsu Inc.). The detection of the light-emission from living bacterial cells was carried out as follows: the plastic Petri-plate cover was removed and replaced with a Petri-plate cover that had approximately 10-20 µL of *n*-decanal (Sigma® Chemical Co., 99% pure) applied over the inside surface of the lid with a cotton swab. The plate was positioned beneath the photon counting camera into the dark low-light imaging chamber and the photons emanating from individual bacterial colonies were counted. *A. tumefaciens* colonies expressing high levels of bioluminescence and displaying vigorous growth on full-selection medium (containing carbenicillin) were then analyzed by restriction enzyme mapping of plasmid DNA to confirm the presence of the plant expression vector pGL101.

II.11 PLANT TRANSFORMATION METHODS

II.11.1 *Agrobacterium*-mediated transformation of *Arabidopsis* roots

The specific procedure and media used for *Agrobacterium*-mediated root transformation are described in Marton and Browse (1991). In this experiment some modifications of the Marton and Browse method were introduced; only the *A. thaliana* ecotype RLD was used; also Gel-rite™ (0.22%) was used as a solidifying agent instead of agar (0.7%). *A. tumefaciens* GV3101 cells, transformed with pGL101, were grown overnight in 25 mL of YEB supplemented with gentamycin (25 µg/mL), kanamycin (50 µg/mL), rifamycin (100 µg/mL) and carbenicillin (100 µg/mL) at 25° C. A 50 µL aliquot of this dense overnight suspension was added directly to excised roots of *A. thaliana* submerged in 10-15 mL of MS liquid medium. Plant roots were first conditioned for 2 days on ARMI medium and were wounded, just prior to the addition of bacteria, by pricking root-tissue with sterile forceps and a sterile scalpel at 1 mm intervals. The roots were incubated in the 10-15 mL of MS medium, exposed to the bacteria, at room temperature for 2 days. After the 2 day incubation period, the roots were washed three times in sterile MS medium and were pulled through solidified MS medium to remove bacteria which were loosely adhering to the surface of the root tissue. After removal of the bacteria, the roots were placed on ARMII medium supplemented with kanamycin (100 µg/ml) for selection of transformed cells and embryogenesis. For selection against *A. tumefaciens*, 300 µg/ml of cefotaxime was used instead of 100 µg/ml each of carbenicillin and cefotaxime prescribed in the protocol. The third rooting medium, ARMIII, was found to have harmful effects on plantlet growth. It did not appear to enhance rooting of plantlets or to enhance shoot development, thus, some plantlets were transferred to MS 1.25% medium supplemented with 50 µg/mL kanamycin and 150 µg/mL cefotaxime for rooting, or plantlets were not excised from the root-sections at the site of embryogenesis and were maintained on ARMII medium.

Twenty-two plantlets which showed some root and/or hairy-rooty callus development were transferred to a potting mix (Metro-Mix 290) in the greenhouse for flowering and seed production. Eight plants yielded sufficient seeds allowing further analysis. The seeds were harvested by collecting the partially dried out seed-stocks into glass jars, allowing the siliques to completely dry, covering the top of the jar with two layers of cheese cloth and then shaking the inverted jar over a large sheet of paper to collect the falling seeds. The seeds were stored in the dark in small plastic scintillation vials at room temperature. Seeds harvested and collected from *Arabidiopsis* plants and stored in this way were viable for two years with no apparent loss in seed germination frequency.

II.11.2 Segregation analysis of kanamycin-resistance

The primary transformed plants (T_o) were allowed to set seed as stated in II.11.1. The seeds (T_1-generation) obtained from the self-fertilized flowers were collected, surface-sterilized and plated onto MS 1.25% medium containing kanamycin (65 µg/mL);

the segregation of kanamycin-resistance of the second (T_1) plant generation was scored. Approximately ten kanamycin-resistant plants from each line were then potted in a potting mix and were transferred to the greenhouse to set seed. The selfed-progeny of the T_1-generation were plated on MS 1.25% supplemented with kanamycin to identify which T_1-plants generated a kanamycin-resistant family of T_2-plants (i.e., homozygous for kanamycin-resistance). The T_2-seeds (i.e., self-fertilized seeds from the T_1-generation), which yielded a family of kanamycin-resistant T_2-plants, were identified from original transformants #2, #3, #4, #5, #6 and #7; these seeds were used to grow homozygous plants for both DNA isolations and for luciferase induction studies.

II.12 ANALYSIS OF TRANSFORMED *ARABIDOPSIS* GENOMIC DNA

II.12.1 PCR analysis of genomic DNA

Total genomic DNA was isolated from putative transformed plants and nontransformed control plants basically as described in II.7. This simple rapid method was scaled down; tissue samples of approximately 0.250 gm (equivalent to one or two small plantlets) were homogenized in CTAB buffer, with a small plastic pestle and sand, in a 1.5 mL Eppendorf tube. DNA isolated in this way was used as the template in a PCR reaction as outlined in II.8.1; this PCR reaction was carried out using both *PAL1* and *lux A* primers. The *PAL1* primers used were the same as noted for the original amplification of the *PAL1* promoter. Two *lux A* primers were chosen which were already in general use in the laboratory and are shown below:

"*lux A* in" (5'-GGAAACTTCCTTCTCACTTATGAG-3')

"*lux A* reverse" (5'-GGCATCACATCAGACTGGAATAGC-3')

These *lux* primers were designed previously (Plant Molecular Genetics, University of Alberta) to amplify approximately a 1.1 kbp *lux A* sub-domain from the monomeric *lux F* reporter gene (Escher *et al.*, 1989).

II.12.2 Preparation of plant genomic DNA

Isolation of plant genomic DNA was based on the method of Doyle and Doyle (1989) with numerous modifications. Two to three week-old *Arabidopsis* plants (10 gm), grown on MS 1.25% medium, were harvested and were homogenized in CTAB buffer (2% [w/v] CTAB, 1.4 M NaCl, 0.2% [v/v] β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8) in a mortar and pestle using 4 mL of buffer/gm of plants. The homogenate was incubated at 60° C for ½-1 hr. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the homogenate and the two phases were mixed briefly for 1 min; this mixture was centrifuged at 8 000-9 000 g for 20 mins to separate the phases and more importantly to clear the aqueous phase of gelatinous polysaccharide debris. The clear, but slightly greenish aqueous phase, was drawn-off and chilled briefly on ice for a few mins. A ⅔ volume of ice-cold isopropanol was added, the two phases were gently mixed and the solution was incubated on ice for 15 mins to precipitate the nucleic acids. The precipitate was centrifuged at 5 000-6 000 g for 10 mins to collect the nucleic acids and the pellet was briefly dried *in vacuo*, taking care not to over-dry. The pellet was dissolved in 1mL of TE followed by the addition of DNase-free RNase A to a final concentration of 30 μg/mL. The preparation was incubated at 37° C for 30-45 mins, 600 μL of 4 M NaCl and 400 μL of 5% [w/v] CTAB were added and two chloroform extractions were carried out. The DNA was precipitated with a ⅔ volume of ice-cold isopropanol, collected by centrifugation in an Eppendorf tube and washed briefly in ice-cold 80% ethanol. This pellet was briefly dried *in vacuo* and was dissolved in TE (pH 7.2). The DNA was quantified and stored as in II.7.

II.12.3 Preparation of Southern blots for genomic DNA analysis

Aliquots of plant genomic DNA (4-5 μg) were digested with *Hind* III/*Bgl*II ("double-digest"), *Bam*HI and *Hind*III using 40-50 enzyme units in a total volume of 100 µL. After 1½ hrs of restriction enzyme digestion, the efficiency of the digestion was examined by electrophoresing a 2-3 µL aliquot of the reaction mixture on an agarose mini-gel. The completely digested DNA was extracted once with chloroform and the DNA was precipitated by adding 30 µL of 4 M NaCl and 2 volumes of 95% ethanol. After pelleting, the DNA was washed briefly in ice-cold 80% ethanol and was dried *in vacuo.* The pellet was dissolved in 20-30 µL of TE and 5 µL of 5 × loading buffer was added (Sambrook *et al.*, 1989); the DNA fragments were separated by electrophoresis through a 1% TAE agarose gel (16 cm × 16 cm), by applying a voltage of 2-2.5 volt/cm for 24-30 hrs. The gel was treated with depurination solution (0.5 M HCl) for 30 mins, transferred to denaturation solution (1.5 M NaCl, 2 M NaOH) for 30 mins and lastly transferred to neutralization solution (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.0) for 15-30 mins. The separated DNA fragments were then blotted onto a Nylon membrane (Amersham Hybond-N) by a vacuum blotting procedure as follows: i) an appropriately gel-sized nylon membrane was placed on the Transvac™ TE-80 blotting tray (Hoeffer Scientific Instruments) overtop a piece of wetted Whatmann® 3mm chromatography paper, ii) the gel was placed "right-side-up" upon the nylon membrane in the blotting-tray-unit and the vacuum was applied using a Red-EVAC™ vacuum (Hoeffer Scientific Instruments) being careful not exceed 5 pounds per square inch of vacuum and iii) the vacuum-transfer proceeded for 1.5-2 hrs, while small aliquots of 20 × SSC (10-20 mL) were poured over-top the gel every 15-20 mins to replace the solution being drawn through the gel by the vacuum, thus preventing the gel from drying-out. After 1.5-2 hrs the gel was removed, the unit was disassembled and the membrane was briefly air-dried. The DNA fragments were "cross-linked" onto the membrane by exposure to UV-light using a UV Stratalinker™ 1800 (Stratagene Inc.) set on the "auto-crosslink" setting. The membrane was then baked for 45 mins at 80° C before hybridization.

II.12.4 Genomic blot hybridization

Nylon membranes were placed in siliconized hybridization tubes (Robbin's Scientific) and approximately 0.150 mL of pre-hybridization buffer (50% formamide, 50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA, 0.9 M NaCl, 10 × Denhardt's (Sambrook *et al*. 1989), 150 μg/mL of salmon sperm DNA and 0.1% [w/v] SDS) were added for every cm^2 of nylon membrane. The hybridization tubes were then placed into the hybridization incubator Model 310 (Robbin's Scientific) and the pre-hybridization was carried out at 42° C for 4-5 hrs. The pre-hybridization buffer was collected into a polypropylene disposable 50 mL Falcon® tube. The denatured radiolabelled *lux F* probe (1×10^8 cpm/μg DNA of ^{32}P in 400 μL of TE) and denatured salmon sperm DNA (final concentration of 250 μg/mL) were carefully added to the pre-hybridization buffer to produce the hybridization solution. The hybridization solution was then carefully mixed and poured back into the hybridization tube. Hybridization was carried out overnight for 16 hrs at 42° C. After hybridization, the hybridization solution was collected and the nylon membrane was washed three times (200 mL each) with washing solution (50 mM NaCl, 1 mM EDTA, 20 mM sodium phosphate buffer, pH 7.0 and 0.1% [w/v] SDS): first for 30 mins at room temperature, then 30 mins at 45° C and finally at 55° C for 30 mins. The membrane was air-dried briefly. The moist membrane was placed onto a cassette sized piece of Whatmann® 3mm paper, wrapped in cellophane and was autoradiographed using a Kodak cassette containing intensifying screens and Kodak X-OMAT™ X-K1 film, at -70° C. X-ray films were developed using an automatic Kodak processor. Nylon membranes were later reused for hybridization with a *PAL1* promoter probe by "striping-off" the previous probe as per the manufacturer's directions and following the identical hybridization procedure above with a new radiolabelled probe.

II.12.5 Preparation of radiolabelled probes

The 2.4 kbp *lux F* (*Hind*III-*Bgl*II) and the 1.8 kbp *PAL1* (*Sal*I-*Bam*HI) fragments, used for probes were excised and isolated from pGL101 and pPAL45 using the indicated restriction enzymes. Following restriction enzyme digestion, the DNA fragments were separated on an agarose gel and the appropriate sized fragments were isolated by electroelution. The DNA was extracted, once with phenol:chloroform (1:1), once with chloroform:isoamylalcohol (24:1) and precipitated with 3 M NaOAc (pH 5.2) and ice-cold 95% ethanol. The precipitate was pelleted, washed briefly with ice-cold 80% ethanol, dried *in vacuo* and redissolved in ddH_2O to a concentration of 30-50 ng/μL.

The procedure for making random-primed radiolabelled probe is based on a procedure outlined by Ausubel *et al.* (1987-1994). The 10 × reaction mixture (2.5 μL) (Boehringer-Mannheim, Random Primed labelling kit) containing 10 × Klenow buffer and random hexanucleotides, was mixed with 100 ng of template DNA and ddH_2O was added to yield 14 μL. This mixture was boiled for 4 mins and was immediately placed on ice. To the denatured and primed template mixture, 2.5 μL of a 3dNTP (minus dATP) stock (0.5 Molar each dNTP) and 5.0 μL (50 μCi) of [α^{32}P]-dATP (3 000 Ci/mM) were added; 2-4 units of Klenow fragment were added to bring the total volume to 25 μL. The reaction mixture was incubated for 4 hrs at room temperature. The unincorporated dNTP's were separated from the radiolabelled probe by using a Sephadex G-50 column (Pharmacia Nick-Column [pre-packed], approximately 3-5 mL bed volume). The probe was eluted into 400 μL of TE, boiled for 4 mins to denature and immediately placed on ice. Before denaturing, a 1 or 2 μL aliquot from this purified probe was examined using a liquid scintillation counter (Beckmann LS3801); the specific activity of the probe was estimated by counting the Cerenkov radiation and relating back to the amount of DNA template used. Using this protocol, the specific activity of the probes prepared in these experiments were typically estimated to be 1-5 × 10^8 cpm/μg DNA.

II.13 ANALYSIS OF PHYTOPATHOGEN INDUCTION OF LUCIFERASE ACTIVITY

II.13.1 Inoculation of *Arabidopsis* leaves with two *P. syringae* pathovars

Four to five week-old plants were grown as described in II.2. One leaf from one plant was chosen and a bacterial suspension was pressure infiltrated through the underside of the leaf using a 1 mL needleless-syringe as described in Dong *et al.* (1991). Neither the young leaves emerging from the top of the rosette, nor the oldest leaves at the very bottom of the rosette were infiltrated. The average sized leaves originating from the middle, or lower-middle of the rosette were generally chosen. *P. syringae*, pathovars *tomato* and *maculicola*, were each grown in 2-4 mL cultures of King's medium B (supplemented with 15 µg/mL nalidixic acid and 150 µg/mL streptomycin, respectively), overnight at 30° C with vigourous shaking. The cultures were washed twice in 10 mM $MgCl_2$ and were re-suspended in 10 mM $MgCl_2$ to a final $O.D._{600}$ of 0.2-0.4, which is equivalent to $1\text{-}2.5 \times 10^8$ cells/mL. According to Dangl *et al.* (1992), a 1 $O.D._{600}$ of *P. syringae* is approximately equal to 5×10^8 cells/mL. One leaf from each transgenic *Arabidopsis* plant was either infiltrated with 5-10 µL of 10 mM $MgCl_2$, *P. syringae* pv. *tomato* (*Pst*), *P. syringae* pv. *maculicola* (*Psm*) suspension, or was left untreated as a control. At the same time, leaves from the control, nontransformed, wild-type *Arabidopsis* plants (ecotype RLD) were infiltrated with *Pst* in a similar way. These control plants were of approximately the same age as the transgenic plants. After leaf-infiltration, plants were incubated in the greenhouse in which they were grown and no changes were made either in lighting or humidity, but after infiltration plants were well watered to insure the soil was visibly and thoroughly wet.

II.13.2 *In vitro* enzymatic assay for luciferase activity

Leaves from transgenic *Arabidopsis* plants were assayed for changes in luciferase activity 24, 48 and 72 hrs after infiltration with the avirulent and virulent *P. syringae* strains essentially as described (Langridge *et al.*, 1989). For a specific treatment a minimum of three leaves from three separate plants were assayed daily for 3 days, beginning 24 hrs after inoculation with bacteria. The infiltrated leaf was removed from the plant and the entire leaf was immediately homogenized in 1 ml of lux buffer (50 mM β-mercaptoethanol, 0.4 M sucrose and 50 mM $Na_2HPO_4*7H_2O$, pH 7.0) in a 1.5 mL Eppendorf tube with a small plastic pestle. A 450 μL aliquot of this homogenate was placed into a glass scintillation vial for photon counting in a luminometer (Turner TD-2e). Meanwhile, a 0.5 mL aliquot of flavin mononucleotide ($FMNH_2$) reaction buffer (100 mM oxidized flavin mononucleotide, 200 mM tricine buffer, pH 7.0, 0.4 M sucrose) was being reduced by incubation in a chamber containing 3 circular florescent lights (Circlite, General Electric). The redox state of $FMNH_2$ can be easily verified with the naked eye since oxidized FMN is a bright yellow colour, while reduced $FMNH_2$ is colourless. The *n*-decanal (20 μL of a sonicated emulsion of decanal/ddH_2O, 1:1000) was then quickly taken up into the syringe containing the 0.5 mL of the light-reduced $FMNH_2$ and the two substrates were co-injected into the sample (already in the luminometer) through a "light-tight" rubber septum; photons were counted for ten seconds and were expressed as the integrated full-value of the light peak in Light Units (1 L.U.= 1.6×10^6 photons/sec.). This procedure was repeated for a second time with another 450 μL of the same leaf homogenate and the two values were averaged to obtain the average L.U. value per 450 μL aliquot of homogenate for that specific leaf sample. The approximately 100 μL of leaf homogenate solution remaining was briefly centrifuged to pellet particulate leaf debris and the clear supernatant was used for comparative total protein determinations. This procedure was carried out for at least three separate leaves from three individual plants per each leaf treatment per assay period. For example, at the 24 hr assay period three leaves each were analyzed in this way from transgenic plant leaves infiltrated with *Pst*, *Psm*, $MgCl_2$ and untreated control (thus totalling 12 leaves from 12 separate transgenic plants). For the nontransformed control *Arabidopsis* plants, only *Pst*-infiltrated and untreated leaves were assayed (totalling at least 6 separate untransformed plants).

II.13.3 Protein Assay

The total protein content from the various leaf homogenates were determined by the Bradford assay (Bradford, 1976), using the BIO-RAD protein assay kit in conjunction with the BIO-RAD bovine serum albumin standard protein solution. The cleared leaf homogenate (10-20 μL) was brought to 0.8 mL in ddH_2O in a plastic cuvette then 200 μL of the BIO-RAD Dye Reagent Concentrate was pipetted into the solution. The A_{595} was measured in the period between 5 mins and 1 hr after the dye concentrate was added.

II.13.4 Monitoring of bioluminescence in transgenic plants, *in planta*

Transgenic plants were grown in the greenhouse as described in II.2. Individual leaves were infiltrated as described previously (II.13.1) and the soil-grown plant (including the pot which contained it) was placed in a 450-mL preserve jar (2½" internal diameter and 2⅓" depth) containing 200 μL of liquid *n*-decanal (99% pure); a large plastic Petri-plate cover was placed over top the jar to contain the *n*-decanal vapours. To determine the light-emission from a specific plant, the jar containing the *Arabidopsis* plant was placed inside the dark low-light imaging chamber (Argus-100 Low-light Imager , Hamamatsu Inc.) to exclude external light. The plant was positioned directly beneath the photon-counting camera and was kept in the dark, inside the low-light imaging chamber for 2 mins to allow for the decay of florescence generated from chlorophyll-containing green leaves. With the low-light imager adjusted to maximum sensitivity, the photon-emission from the plant was captured in "real-time" for a period of 10-12 mins. The image of bioluminescence from the plant was than superimposed upon a video image of the same plant, thereby localizing the site of strong light-emission. After photon collection, the plant was immediately removed from the decanal vapour-filled jar and was returned to the greenhouse for further measurements the following day. Monitoring of luminescence from infected leaves was performed on plants which were grown under the identified greenhouse conditions (II.2) and which were receiving adequate water and light. To minimize any possible effects of irrelevant environmental stresses, other than microbial infection, on light-emission, plants were placed in the imager only during the day and shortly before luminescence determinations. Plants were assayed once a day, for a period of 3-5 days. Using the Argus 100 Low-Light Imager software (Argus 100 Version 3.00 Photonic Microscope System), the total number of photons emanating from an infiltrated leaf, or even from a specific area on a infiltrated leaf, could be determined. Specifically, the luminescence emanating from small, circular, pin point-sized sectors (5% of the total leaf area) was calculated by the data analysis program.

II.14 DETECTION OF LUX F PROTEIN IN TRANSGENIC PLANTS

II.14.1 Preparation of plant tissue homogenates

Plant tissue samples (0.200-0.250 gm) were taken from the base of the inflorescence of transformed and nontransformed control flowering plants grown under identical conditions as stated in II.2. The tissue samples were homogenized in a mortar on ice, with a pestle and sand, in an aliquot of ice-cold grinding buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF and 1% Triton X-100). Initially, grinding buffer was added in 1:1 ratio (weight of tissue/volume of buffer) to the tissue, however, if the consistency of the homogenate was too viscous, additional volumes of grinding buffer were added to a maximum ratio of 1:3 w/v. This homogenate was centrifuged in a microcentrifuge at the highest speed (12 000 rpm) at 4° C for 15 mins and the cleared supernatant fraction (150-400 μL) was pipetted-off into another 1.5 mL Eppendorf tube. To this supernatant, 40-100 μL of 5 × sample buffer (10% glycerol, 2% [w/v] SDS, 0.125% [w/v] bromophenyl blue, 0.5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) was added and the sample was boiled for 3 mins.

A Lux F protein-containing homogenate was prepared as a positive control. The positive control was prepared in the laboratory (Plant Molecular Genetics, University of Alberta) and consisted of *E. coli* DLT-101 cells carrying the plasmid pLX703 fab-2 (Escher *et al.*, 1989); cells were grown to an O.D.$_{600}$ of 1.0 in LB medium supplemented with ampicillin (100 μg/mL). High levels of Lux F protein synthesis were induced by the addition of IPTG (final concentration 0.4 mM) to the cell culture. After a 1 hr induction period, 100 μL aliquots of the cell suspension were centrifuged in the microcentrifuge and the supernatant was drawn-off. The bacterial pellet was then stored at -20° C for later use. Before loading the gel, the *E. coli* cells were re-suspended in 50 μL of 1 × sample buffer (referenced above) and boiled for 3 mins. Total protein samples from the plant tissue and from the bacterial positive control were now ready for separation by way of SDS-PAGE.

II.14.2 SDS-PAGE and blotting the proteins

A 3 mm thick, 16 cm × 16 cm, SDS-polyacrylamide gradient gel (7.5%-20%) with a 4% polyacrylamide stacking gel was prepared using a gradient mixer and according to standard procedures (Protean® II Slab Cell Instruction Manual, BIO-RAD). Before adding sample buffer to the cleared supernatant fraction (II.14.1), the concentration of proteins was determined using the BIO-RAD Protein Assay as in II.13.3; 70, 140 and 240 μg of both the sample and the negative control (nontransformed plant tissue) were loaded onto the gel, along with 20 μL of the bacterial-lysate positive control.

The electrophoresis was carried out overnight (18 hrs) at a constant current of 35 mAmps., employing the running buffer specified in the Protean® II Slab Cell Instruction Manual (BIO-RAD). After protein separation, the gel was removed from electrophoresis chamber and the proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (BIO-RAD) using a semi-dry electroblotting transfer unit (Semi-phor™, Hoeffer Scientific Instruments). Transfer was carried out as recommended by the manufacturer at a constant amperage of 200 mAmps. for 3 hrs. After transfer was complete the PVDF membrane was allowed to dry at room-temperature. The gel was immersed in 25% isopropanol and 10% acetic acid for 1 hr, stained in staining solution (0.2% coomassie blue dye, 45% methanol and 7% acetic acid) for 1 hr and de-stained for 1 hr (45% methanol and 7% acetic acid) to visualize the proteins remaining in the gel.

II.14.3 Immunoblotting Procedure

The dried PVDF membrane, bearing the transferred proteins, was first moistened in 100% methanol for 5 mins, then rehydrated in dH_2O for 5 mins, followed by incubation for 5 mins in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20). The membrane was then treated in TBST containing 1-1.5% BSA and 20% fetal calf serum for 1 hr before addition of the first antibody solution. The blocking solution was replaced with TBST containing the primary polyclonal rabbit Ab: anti-Lux AB protein (prepared by K. Illes, Plant Molecular Genetics, University of Alberta), at a dilution of 1:10 000. The membrane was incubated with shaking for 1 hr followed by three washes (5-10 mins each) with TBST. In the following step, the membrane was incubated with shaking in TBST containing the secondary goat IgG Ab: anti-rabbit-alkaline phosphatase antibody- conjugate (Sigma® Immunochemicals), at a 1: 8 000 dilution for 30 mins. The membrane was then washed three times with TBST (5-10 mins each) and was incubated in Alkaline Phosphatase buffer (10 mM Tris-HCl, pH 9.5, 100 mM NaCl and 3.5 mM $MgCl_2$) for 2 mins. A Sigma FAST™ Bcip/Nbt buffered substrate tablet (Sigma® Immunochemicals) was dissolved in 10 mL of ddH_2O by vortexing. The 10 mL of the substrate solution was poured into a suitable container and the PVDF membrane was carefully layered on the bottom of the container ensuring that the whole membrane was immersed in the substrate solution. The reaction was allowed to proceed in the dark for 5-10 mins. When sufficient colour had developed the substrate solution was poured-off. The membrane was washed briefly in dH_2O, left to air-dry and stored in the dark at room-temperature.

III. RESULTS

III.1 PCR AMPLIFICATION AND CLONING OF THE *ARABIDOPSIS PAL1* PROMOTER

The isolation of total *Arabidopsis* genomic DNA was required for cloning of the *Arabidopsis PAL1* promoter (Ohl *et al.*, 1990) using PCR. Total genomic DNA was isolated from *A. thaliana* (ecotype *Landsberg erecta*) plants. DNA from this ecotype was used as the template since the *PAL1* promoter was originally cloned from this ecotype of *A. thaliana* and it was unknown whether there might be significant differences in the *PAL1* promoter sequences between the different *Arabidopsis* ecotypes.

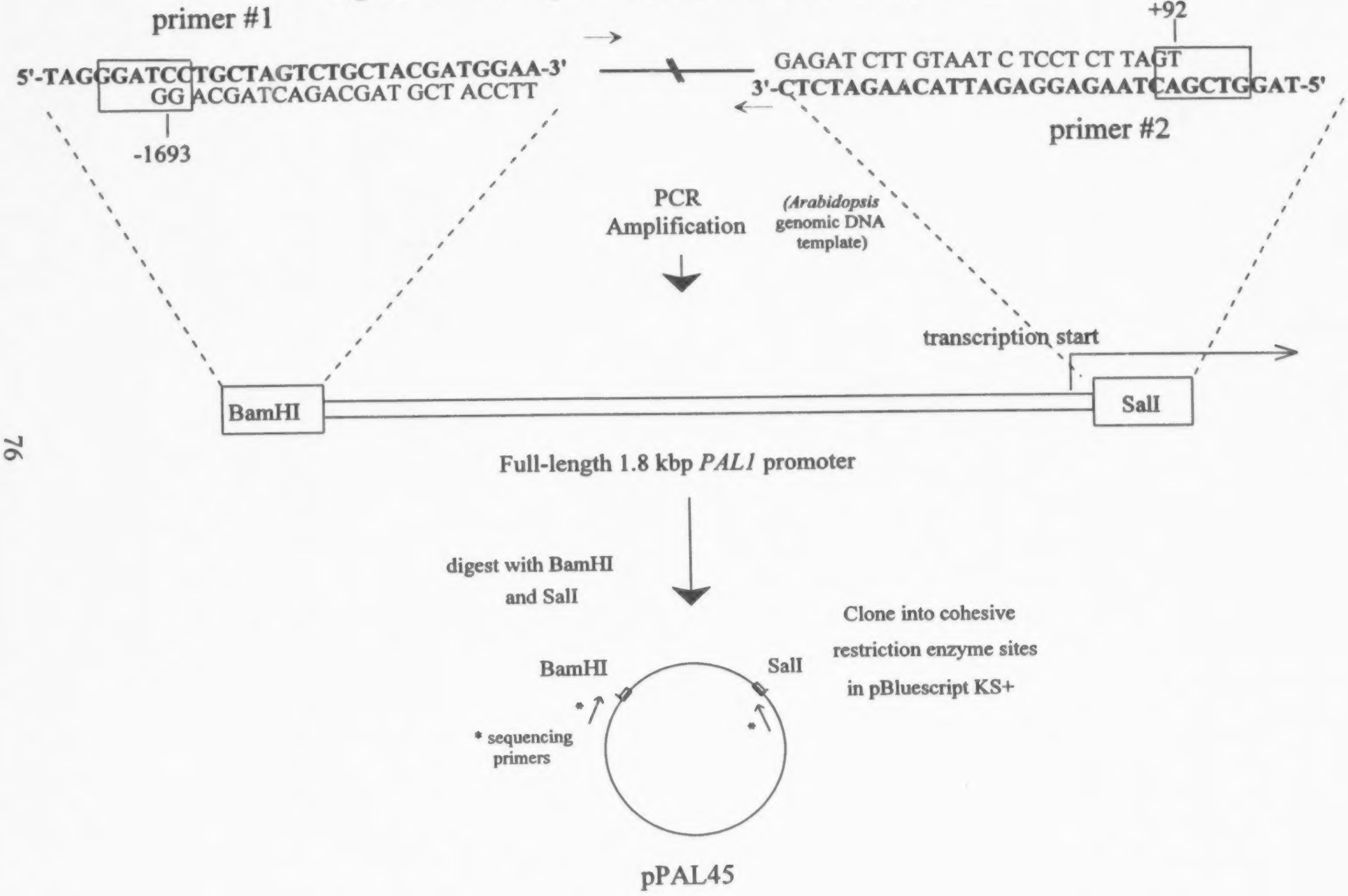
The haploid genome size of *Arabidopsis* was estimated to be approximately 0.073 picograms (70 Mbp, (Meyerowitz, 1989) and 965 Mbp of DNA= 1 picogram). Two hundred nanograms of *Arabidopsis* genomic DNA is approximately equal to 2×10^6 genomes. Therefore, 200 ng contains approximately 2×10^6 copies of the target sequence and provides an abundant number of original target molecules that should be amplifiable in a minimum of cycles; any number of target molecules greater than 1×10^5 should be easily amplifiable (Saiki, 1990). PCR is so sensitive that a single DNA molecule has been amplified and single-copy genes are now routinely extracted and amplified out of a complex mixture of genomic sequences and visualized as distinct bands on agarose gels (Innis and Gelfand, 1990). The number of target molecules is recommended to be between 10^5 and 10^6 ; when DNA of a known concentration is available amounts of 0.05 to 1.0 μg are typically used for the amplification of a single-copy locus from genomic DNA (Saiki, 1990). Hence 200 ng of total genomic DNA and 50 pmol (approximately 3.0×10^{13} molecules) of each primer were used in a total reaction volume of 50 μL.

Based on published sequence data (Ohl *et al.*, 1990), two primers were designed to anneal to and to amplify the full-length *PAL1* promoter from nucleotide -1693 to nucleotide +92 with respect to the putative transcription initiation site. The primers were also designed to incorporate a unique *Bam*HI and a unique *Sal*I restriction endonuclease site at either termini of the full length PCR product (see Fig. 2), to permit direct cloning into the plant expression vector pPCV701*luxFM1*. These two regions of genomic DNA were chosen for primer annealing because both regions have no identified conservation to other genomic sequences, therefore, primer annealing will be specific resulting in only *PAL1* promoter amplification. These regions of DNA are not translated and are not known to be involved in promoter regulation. The downstream region selected was located well past the putative transcription start site to ensure that the transcription initiation site was included in the full-length PCR product and thus promote efficient transcription initiation once reintroduced into the plant. A 9 base-pair sequence located in the 5' untranslated leader region (nucleotides +22 to +31) of the *PAL1* gene demonstrated high homology to regions in other phenylpropanoid biosynthetic genes and therefore may be an important promoter regulatory element (Ohl *et. al.*, 1990); another conserved sequence element was identified (-1595 to -1607) far upstream of the transcription initiation and was also considered to be an important promoter element. Thus the PCR primers were designed to amplify the "full-length *PAL1* promoter".

Based on the designed PCR primers a PCR product of 1.8 kbp was expected. In fact the 1.8 kbp PCR product was clearly visible on a EtBr-stained agarose gel; it was the expected size and no additional products (e.g., primer-dimer) were visible indicating a successful specific PCR amplification reaction with relatively high yields (about 1.00 μg DNA/50 μL reaction preparation).

The PCR product was isolated from a TBE agarose gel using Geneclean® and was digested sequentially with *Bam*HI and *Sal*I. The restriction enzyme-digested PCR product was again gel-purified and was added to *Bam*HI-*Sal*I digested pBluescript KS+ in a 1:1 vector to insert ratio. To test for the re-ligation of the vector a separate control

Figure 2: Cloning of the *Arabidopsis PAL1* promoter


primer #1
5'-TAGGGATCCTGCTAGTCTGCTACGATGGAA-3'
GG ACGATCAGACGAT GCT ACCTT
-1693
+92
GAGAT CTT GTAAT C TCCT CT TAGT
3'-CTCTAGAACATTAGAGGAGAATCAGCTGGAT-5'
primer #2
PCR
Amplification
(Arabidopsis genomic DNA template)
transcription start
BamHI
SalI
Full-length 1.8 kbp PAL1 promoter
digest with BamHI and SalI
Clone into cohesive restriction enzyme sites in pBluescript KS+
BamHI
SalI
* sequencing primers
pPAL45

reaction, containing the same reaction components minus the PCR product, was used. α-Complementation, otherwise known as "blue/white colony selection," was used to isolate bacterial transformants which possessed the pBluescript KS+ vector containing the PCR-generated *PAL1* promoter insert. The pBluescript KS+ vector contains the nucleotide sequences designated *lacZ'*, which encodes the first 196 amino acids of β-galactosidase and can complement bacterial host-cells which contain the carboxy terminal portion of the protein (Sambrook *et al.*, 1988). The "Lac-positive" bacteria that result from α-complementation are easily recognizable because they can form blue colonies in the presence of the chromogenic substrate X-gal. Insertion of foreign DNA into the multiple cloning site in the *lacZ'* sequences disrupts the *lacZ'* gene and usually results in the production of an amino terminal fragment that is not capable of α-complementation resulting in" Lac-negative" white bacterial colonies.

Plating 10% and 90% of the bacterial transformation mixture (containing the PCR insert), resulted in 7 colonies (6 white and 1 blue) and 96 colonies (38 white and 58 blue), respectively. All the white colonies from the ligation mixture containing the insert were examined by miniprep. plasmid isolation followed by restriction enzyme analysis with *Bam*HI and *Sal*I; no inserts were detected. The procedure was repeated, but instead of a 1:1 vector to insert ratio, a 1:5 vector to insert ratio was used. This bacterial transformation mix was plated-out (10% and 90%) on LB agar medium supplemented with ampicillin (100μg/mL) and it yielded 27 colonies (all white) and 240 colonies (5% blue), respectively; while the control transformants (without the addition of PCR insert) generated 10 colonies (all white) and 160 colonies (50% blue), respectively. White colonies (54) from the sample reaction were examined by miniprep. isolation of plasmid DNA followed by restriction enzyme analysis. Three colonies revealed inserts. One of the plasmids displayed a 1.8 kbp *Bam*HI-*Sal*I fragment and therefore probably contained the *Arabidopsis PAL1* promoter; this vector was designated pPAL45.

The termini of the insert were sequenced using -21m13 Universal and Reverse primers and the sequence data generated, approximately 250 of nucleotides from either terminus, confirmed that the *PAL1* promoter was indeed cloned. The large excess amount of PCR product, to amount of vector, required for the successful cloning of the PCR product, indicates that the termini of the product may be digested by these restriction enzymes at a low efficiency. Not all restriction endonucleases can cleave with high efficiency at the termini of DNA fragments. According to New England Biolabs (Product Catalogue, 1990-1991) *Bam*HI can cleave with >90% efficiency within two nucleotides from the terminus of a DNA fragment. No information with respect to the efficiency of *Sal*I cleavage near the termini of DNA fragments was available. It should be noted that the additional three nucleotides incorporated between the termini and the restriction enzyme site of the PCR product were designed into the PCR primers with this concern in mind.

III.2 SEQUENCING OF THE *PAL1* PROMOTER

A series of nested deletions were made in the *PAL1* promoter using Exonuclease III and S1 nuclease. Digesting pPAL45 with *Bam*HI resulted in a Exonuclease III "susceptible" 3' recessed-terminus at the *PAL1* promoter, at nucleotide -1693 with respect to transcription initiation. Subsequent digestion of pPAL45 with *Sac*I produced an adjacent "protected" 3' protruding-terminus, on the border of the multiple cloning site leading into the vector. Deletions were made into the *PAL1* promoter using Exo III/S1 digestion, commencing at nucleotide -1693 on the "susceptible" *Bam*HI site. Through restriction enzyme analysis, a set of eight clones were isolated which appeared to contain 200-300 bps, consecutive, overlapping deletions and hence, would cover the entire length of the *PAL1* promoter (200-300 bps × 8 = 1600-2400 bps). One strand of the *PAL1* promoter was sequenced using Sequenase® sequencing kit, -21m13 Universal primer and [^{35}S]-dATP. This sequence analysis data, when compared to the published sequence data of the *PAL1* promoter, confirmed that 800 nucleotides of the cloned *PAL1* promoter sequence were correct and contained no misincorporations.

Further sequencing was carried out using an ABI model 370A automated DNA sequencer equipped with an Apple IIc Macintosh computer loaded with ABI model 373 DNA sequencing software. The eight individual plasmids were sequenced, several times each, generating clear data often up to 400 nucleotides in length per sequencing run. This sequence data, which was compared to the published sequence data, revealed that not a single nucleotide mutation had been introduced by the PCR reaction (see Fig. 3).

Based on this sequence data I feel quite confident that no misincorporations occurred during PCR amplification. Also, the *Taq* polymerase and the PCR reaction have been shown in several instances to be of extremely high fidelity. For instance in one case Fucharoen *et al.* (1989) amplified and cloned the entire β-globin genomic region. The entire sequence of the 30 cycle amplified mammal β-globin gene was sequenced for five randomly picked clones and was determined (14999 nucleotides in total). No errors were detected, thus, the cumulative error frequency and the average mutation rates were calculated to be 6.6×10^{-5} and 5×10^{-6} errors, per nucleotide incorporated per cycle, respectively. There are other reports verifying the high fidelity of PCR; these reported cases typically utilize lower dNTP and Mg^{2+} ion concentrations (200 mM each dNTP and 1.5 mM $MgCl_2$), which are similar to concentrations used to amplify the *PAL1* promoter in this study (200mM each dNTP and 2.5 mM $MgCl_2$) (Gelfand and White, 1990). Other important features of high fidelity PCR reactions are the use of a minimum number of cycles (25-30 cycles) and of selective high annealing temperatures, both of which were

Figure 3: A segment of the nucleotide sequence (-459 to -143) of the *Arabidopsis PAL1* promoter. Sequence data was generated by using a *Taq* cycle-sequencing method and dye-labelled -21m13 Universal primers. An ABI automated DNA sequencer model 370A in conjunction with ABI 373A DNA Sequencing software (version 1.1.1), were used to detect and analyze the fluorescent, dye-labelled, DNA sequencing products. Nucleotides 1 to 18 are pBluescript KS+ sequences. In this particular sequencing run of a deletion derivative of pPAL45 (#6), nucleotide 19 corresponds to nucleotide -459 of the *PAL1* promoter (Ohl *et al.*, 1990). Nucleotide sequences 45 to 56 and 121 to 132 correspond to conserved regions -440 to -429 and -396 to -385 identified by Ohl *et al.*, and are, in addition to other regions, implicated in the control of this promoter. Eight deletion derivatives of pPAL45 were generated from the 5' terminus of the *PAL1* promoter: derivative #1 commenced at -1546, #2 at -1247, #3 at -991, #4 at -731, #5 at -572, #6 at -459, #7 at -326 and #8 at -172. The overlap or irregularity of peaks at the end of each row of data is due to an incompatibility between the printer and the printer driver software.

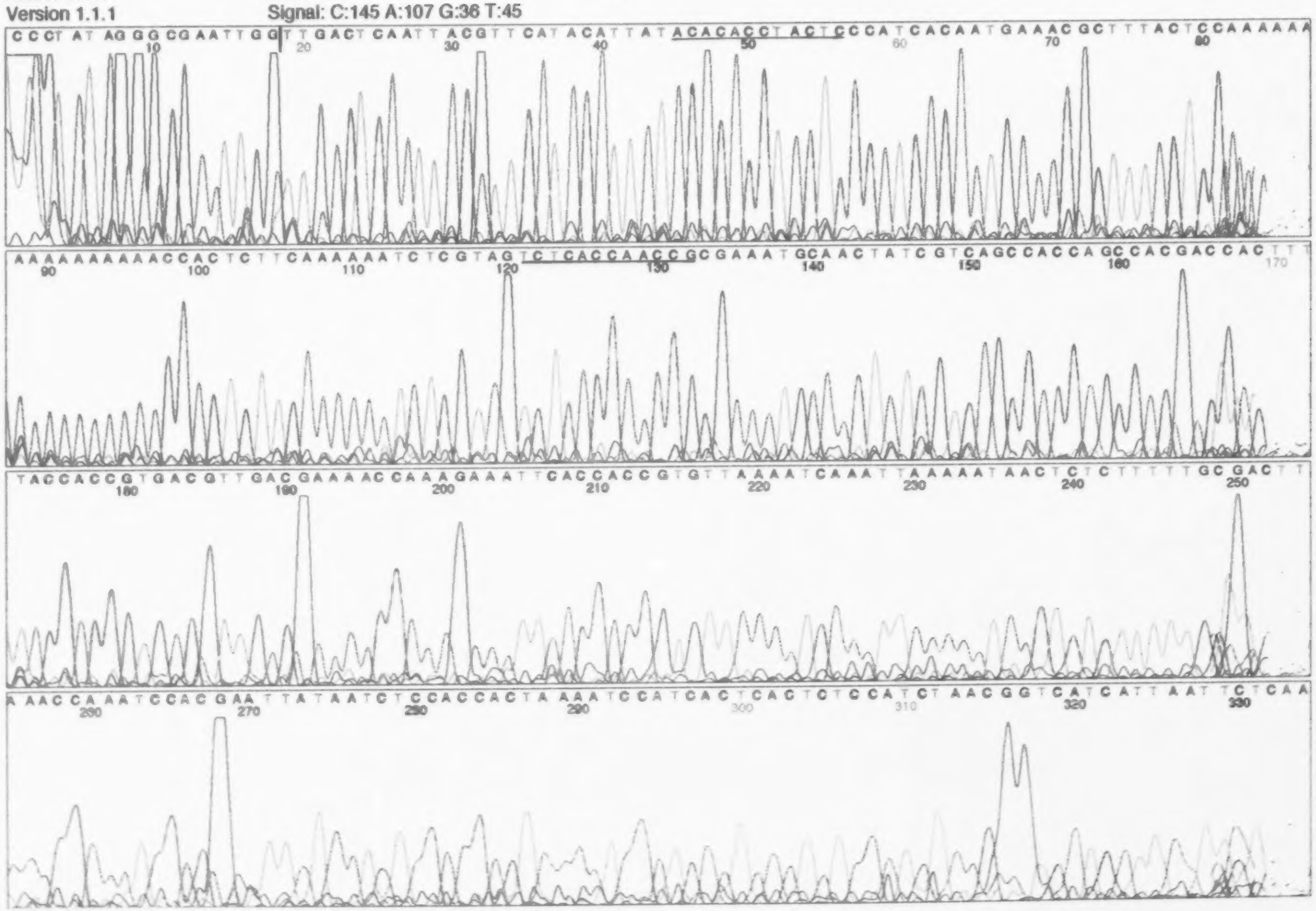
Applied Biosystems
Model 373A
Version 1.1.1

Sample-LORI 12
DyePrimer{-21m13}
Lane 12
Signal: C:145 A:107 G:36 T:45

Points 794 to 5500 Base 1: 794
SN MATRIX FILE 1/05/93
1000

Wed, Jul 14, 1993 4:47 PM
X: 0 to 5678 Y: 0 to 1200

Page 1 of 2



also employed in this study.

III.3 CONSTRUCTION OF VECTOR pGL101 FOR TRANSFORMATION INTO *AGROBACTERIUM*

The *mas* promoter, from the plant expression vector pPCV701*luxFM1*, was excised using *Sal*I and *Bam*HI. The *Sal*I-*Bam*HI *PAL1* promoter fragment was isolated from pPAL45 and was subsequently ligated into the cohesive *Sal*I-*Bam*HI sites generated in pPCV701*luxFM1*. The ligation mixture was transformed into *E. coli* DH5α cells and the transformants were analyzed by restriction enzyme digestion of plasmid. One-third of the plasmids isolated contained the *PAL1* promoter insert into the pPCV701 plasmid, forming a *PAL1-lux F* transcriptional fusion. This novel 13.2 kbp plant expression vector depicted in Fig. 4 was named pGL101(*PAL1-lux F*).

The plasmid pGL101 was transformed into competent *E. coli* S-17 cells. *E. coli* S-17 cells bearing pGL101 were then used to transform *A. tumefaciens* GV3101 by way of conjugative transfer. Transconjugant *A. tumefaciens* cells were isolated by : i) selection on the full complement of *Agrobacterium* antibiotics, including carbenicillin (listed in the Materials and Methods, II.2), ii) by detection of luminescence, *in vivo*, using a low-light imager and iii) by restriction enzyme analysis of miniprep. plasmid DNA. Several colonies were isolated which showed good growth on YEB medium containing carbenicillin. These colonies also exhibited extremely high levels of bioluminescence, several orders of magnitude greater than background, which were detected *in vivo*, upon exposure to *n*-decanal vapour. The presence of the *lux F* gene in some cloning procedures, or in this case a conjugation analysis, was found to be quite useful. In this instance the success of the conjugation was easily determined by the examination of transconjugants *in vivo* using a low-light imager, which quickly revealed the presence of the *lux F* gene in the transconjugants, since untransformed *A. tumefaciens* cells normally

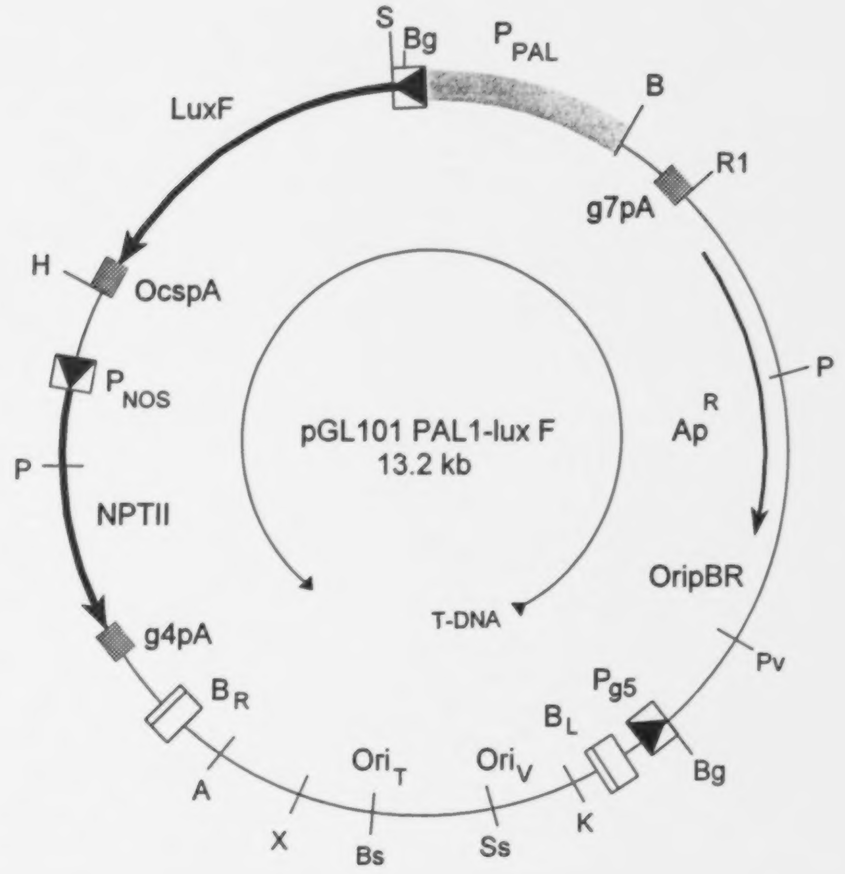

S
Bg
P PAL
B
R1
g7pA
LuxF
H
OcspA
P NOS
P
NPTII
pGL101 PAL1-lux F
13.2 kb
Ap R
OripBR
T-DNA
g4pA
B R
Pg5
B L
Bg
Pv
Ori T
Ori V
A
X
Bs
Ss
K

Figure 4: The plant expression vector pGL101(*PAL1-lux F*) carrying the *lux F* reporter gene fusion (*lux AB*) under transcriptional control of the *A. thaliana PAL1* promoter (P_{PAL}). The T-DNA between the right and left borders (B_L and B_R) indicates the transferred DNA which is stably introduced into the plant genome. The arrows indicate the direction of transcription; P_{NOS}, the nopaline synthase promoter; Pg5, the promoter of the T_L-DNA gene5; g4pA, OcspA and g7pA are all poly-adenylation sequences derived from T_L-DNA gene4, the octopine synthase gene and gene7; NPTII, the neomycin phosphotransferase gene; Ori_T and Ori_V the replication and conjugal-transfer origin sequences derived from plasmid pRK2; oripBR, replication of origin of pBR322; Ap^R the *bla* gene conferring resistance to ampicillin; kb, kilobases. Restriction enzyme sites: A, *Apa*I;B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Ss, *Sst*II; X, *Xho*I.

only show background levels of luminescence.

III.4 TRANSFORMATION OF *ARABIDOPSIS* WITH *AGROBACTERIUM* CARRYING THE VECTOR pGL101

A. tumefaciens cells transformed with plasmid pGL101 were grown overnight and added to roots of *A. thaliana* (ecotype RLD) in MS liquid medium. Ecotype RLD was chosen for transformation since the RLD ecotype is recognized to respond favourably to tissue-culture conditions and is transformed by means of *Agrobacterium* with a higher efficiency than other *Arabidopsis* ecotypes (Marton and Browse, 1991; Koncz *et al.*, 1992). Two to three weeks after plant cell transformation, small, bright-green, thinly scattered calli began to form and were clearly visible on the bleached root-tissue (see Fig. 5). Several days after callus formation, dark-green pinpoint-like "spots" formed centrally on the small, green, globular calli. Upon closer examination of the tissue using a dissecting microscope, the dark-green "spots" were identified to be plant embryos in the torpedo stages of embryogenesis.

In a total of eight Petri plates containing 2-3 intact root sections (segments) each, approximately 100 independent embryos were seen. Some root-sections showed no callus or embryo formation and appeared to be nonviable even before the transformation procedure began, while those root sections that appeared healthy before the transformation procedure began usually gave high frequencies of embryo formation. Some root-sections, which were not exposed to *A. tumefaciens*, were used as the negative control and showed extensive bleaching with no callus or embryo formation. The embryos continued to form in the following 2-4 weeks into small, green, healthy plantlets.

Plantlets were excised and removed onto ARMIII medium for rooting, however, this medium was observed to have a generally detrimental effect on the growth of the

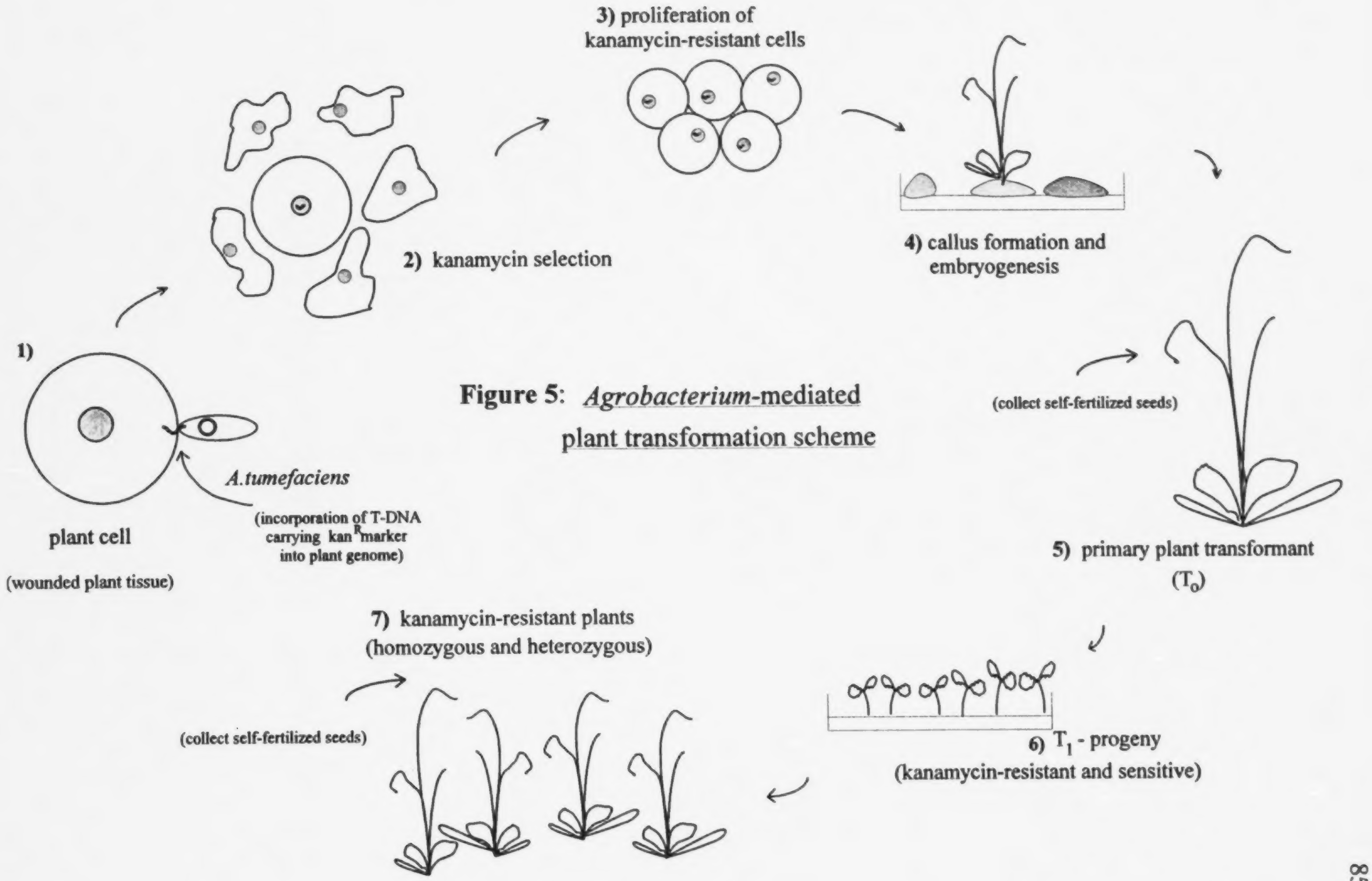

1)
plant cell
(wounded plant tissue)
A.tumefaciens
(incorporation of T-DNA carrying kan^R marker into plant genome)
2) kanamycin selection
3) proliferation of kanamycin-resistant cells
4) callus formation and embryogenesis
(collect self-fertilized seeds)
5) primary plant transformant (T_0)
6) T_1 - progeny (kanamycin-resistant and sensitive)
(collect self-fertilized seeds)
7) kanamycin-resistant plants (homozygous and heterozygous)

Figure 5: *Agrobacterium*-mediated plant transformation scheme

plantlets and definitely did not stimulate root-formation. The majority of plants placed on this medium exhibited reddening of plant leaf tissue, spontaneous premature flowering and downward leaf-curling which raised the plantlets up of the medium; all these responses are characteristic of needless and damaging stress. Consequently, about one-third of the total plantlets were placed on this medium while one-half of the remaining plantlets were placed on MS 1.25% supplemented with kanamycin and cefotaxime for rooting; the remaining one-fifth were not excised, but were left on the bleached root segments on whose surface they had formed and were maintained on ARMII. No plantlets formed roots on ARMIII medium while several plantlets formed roots on MS 1.25%. The plantlets which were left on root-sections and maintained on ARMII medium actually formed roots at a higher efficiency than the preceding two rooting methods. In total, 22 plantlets showed notable root and/or hairy-like callus development on the bottom of the stem where the major root shoot should originate. After 5-6 weeks all of the approximately 100 plants were transferred to the greenhouse and were potted in Metromix potting soil for flowering and seed formation.

Many plants flowered followed by silique formation. Nevertheless, these seed pods were generally empty or contained small, shrivelled, nonviable seeds. It was hoped that the hairy root-like formations would be sufficient for plant survival, plant growth and seed formation as noted elsewhere (Redei *et al.*, 1988) and that plantlets might be stimulated to form roots. However, the poor root development and/or the shock of transfer from the *in vitro* tissue culture conditions to the greenhouse, proved to be generally inhibitory to the growth of the plants. Only 8 primary plants (T_o) survived and set viable seeds in a sufficient quantity that could be further examined.

Seeds from the 8, primary, putative, transgenic plants (T_o) were collected, surface-sterilized and plated onto sterile kanamycin-selection medium. In this way the T_1-

progeny could be screened and provide initial confirmation as to the success of the transformation procedure. The number of kanamycin-resistant and kanamycin-sensitive seedlings of the putative transgenic lines are listed in Table 1. Wild-type non-transformed RLD seedlings were all sensitive to kanamycin and bleached and died within two weeks; while the T_1-progeny from #1, #2, #3, #4, #5, #6, and #7 yielded both healthy, green, kanamycin-resistant seedlings and kanamycin-sensitive seedlings. The calculated chi-square values of the ratio of sensitive to resistant seedlings indicate that #2, #3, #4, #5, each apparently contain one dominant Mendelian-locus for kanamycin-resistance, while #6 likely contains two kanamycin-resistance loci. Progeny from transformant #1 yielded a large excess of kanamycin-sensitive seedlings indicating that the initial transformant may have been a chimaera originating from possibly one transformed kanamycin-resistant cell and several nontransformed, kanamycin-sensitive "escapee" cells. Progeny of primary transformant #8 were all kanamycin-sensitive, hence #8 was labelled a"false positive" and its progeny were not examined further.

It has been shown that a population of T-DNA transformed *Arabidopsis* plants shows segregation for the kanamycin-resistance marker and that usually each primary transformant is segregating for one or more inserts. The previous testing of more than 800 transformed lines indicated that the average number of functional inserts or loci per transformant is 1.4; nearly all transformants are hemizygous. Interestingly, insertions at a single locus may contain dimeric or trimeric T-DNA inserts in up to 60% of the transformants depending on the type of T-DNA vectors, Ti-plasmid virulence genes and transformation technique used (Spielmann and Simpson, 1987; Feldmann, 1992; Grevelding *et al.*, 1993).

Approximately ten kanamycin-resistant T_1-plants from each line were placed in the greenhouse for flowering and seed-collection. Theoretically, from a population of ten

Table 1: The segregation ratio of kanamycin-resistance in the T_1-progeny from eight individual *Arabidopsis* plants transformed with the vector pGL101. The primary transformant plants (T_o) were self-fertilized and the progeny were tested for the segregation of the kanamycin-resistance phenotype by plating seeds on MS 1.25% medium supplemented with 65 µg/mL kanamycin.

Transgenic Line	Total Seedlings Counted	Kanamycin Sensitivity (resistant : sensitive)		$\chi 2^b$	P	Estimated No. of Kanamycin-Resistance Loci
		observed	suggested[a]			
1	66	16:50	1:3	90.7	<0.1	N.A.
2	162	118:44	3:1	0.403	~0.40	1
3	140	107:40	3:1	0.360	~0.55	1
4	137	105:32	3:1	0.147	~0.66	1
5	164	126:38	3:1	0.366	~0.55	1
6	144	144:11	15:1	0.474	~0.45	2
7	13	12:1	---	---	---	N.A.
8	36	0:36	---	---	---	0

[a]- the suggested (theoretical) ratio derived from the observed data is given in this column

[b]- the observed ratio was compared to the nearest suggested (theoretical) ratio by a $\chi 2$ test. At P=0.05 non-significance for $\chi 2 < 3.84$

T_1-plants, derived from a self-fertilizing, hemizygous, primary transformant, one third (approximately 3 plants from the 10) should be homozygous for kanamycin-resistance; this applies in the case of a hemizygous primary transformants exhibiting Mendelian-segregation in a single kanamycin-resistance locus. Transformant #6, which appeared to contain two Mendelian-loci, would require a larger population to isolate an offspring which is homozygous in both of the loci, however, it would be impossible to ascertain whether one has actually isolated an individual offspring plant that was truly homozygous in both kanamycin-resistance loci. An individual plant which is homozygous in one kanamycin-resistance locus, but heterozygous in the other locus, will still yield all kanamycin-resistant progeny. Nonetheless, the same procedure of planting ten plants in the greenhouse was performed allowing a high probability of isolating some progeny that would be homozygous in at least one locus.

The seeds from the family of 10 individual T_1-plants, originating each from an individual transformant, were collected, surface sterilized and plated on MS 1.25% containing kanamycin. As recorded in Table 1, 75% of the T_1 progeny obtained from the self-fertilization of the primary transformants were kanamycin-resistant (excluding #1, #6, #7 and # 8). These kanamycin-resistant T_1-progeny were either homozygous or hemizygous for the inserted T-DNA. The homozygous T_1-plants from each line were identified since they produced only kanamycin-resistant T_2-progeny; thus the T_1-lines that were homozygous for the transgene were chosen by the phenotype of the T_2-generation. The seeds from homozygous T_1-plants, which produced all kanamycin-resistant progeny, were collected and were used in all further studies. When seed-stocks from a particular homozygous line were low, they were replenished by self-pollination of homozygous T_1-plants. The seeds collected from homozygous T_1-offspring plants (originating from the hemizygous primary transformants #2, #3, #4, #5, #6 and #7), were amplified and were stored as source of plants for genomic DNA isolation and for bacterial induced luciferase activity studies.

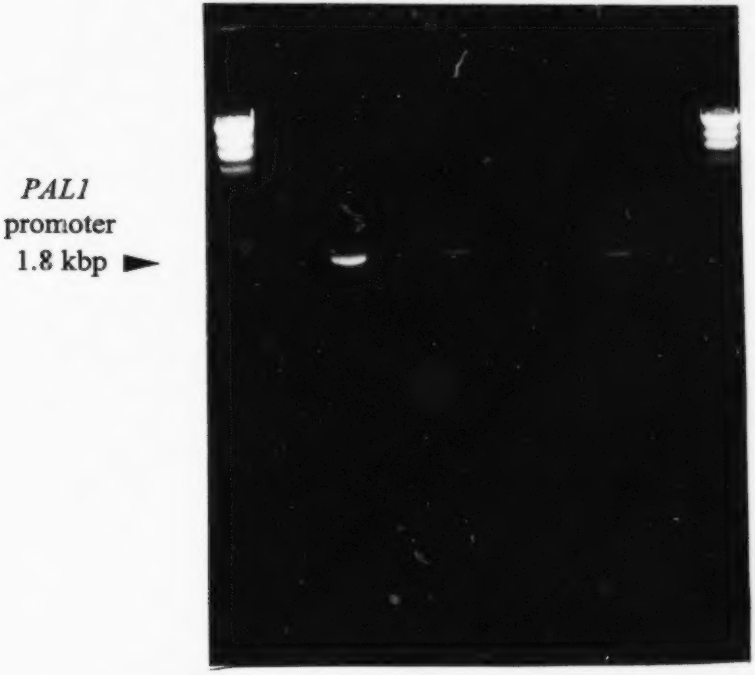
The kanamycin-segregation analysis and selection for homozygous plants was performed in the described manner to allow an initial evaluation to determine if the plants were in fact transgenic and if so, to determine the number of kanamycin-resistance loci. Also, the selection and isolation of homozygous plants proves indispensable when measuring luciferase activity in plants, since gene-dosage effects could introduce differences in luciferase reporter enzyme measurements between plants which might reflect the reporter gene number and consequently will not be indicative of reporter gene regulation or promoter induction. Phenotypically, all the transformed plants used in this study appeared normal; no aberrant identifiable phenotypes were noted in the transformed *Arabidopsis* plants.

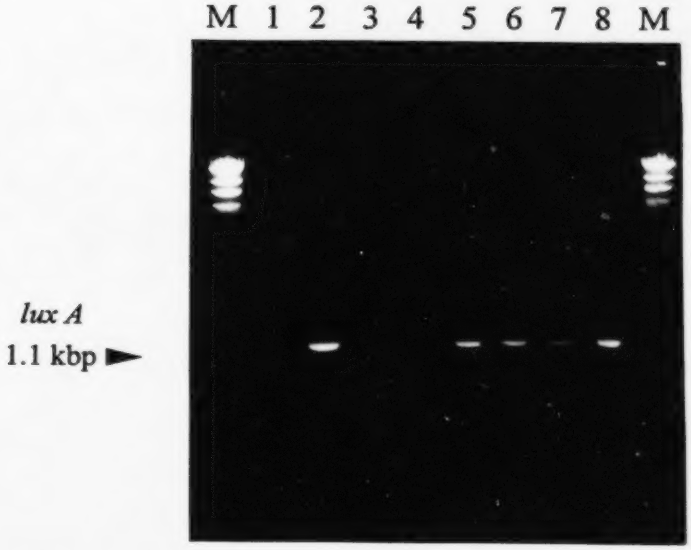
III.5 CHARACTERIZATION OF THE GENOTYPES OF FOUR TRANSGENIC LINES OF *ARABIDOPSIS*

Initially attempts were made to characterize the genotypes of four kanamycin-resistant, putative, transgenic lines by using PCR. Plant lines #2, #3, #5 and #6, were chosen and genomic DNA was isolated using a scaled-down procedure of Doyle and Doyle (1990). Both *PAL1* primers and *lux A* primers were used; the *PAL1* primers provided a positive control since all samples including DNA from the wild-type plants should possess the *PAL1* promoter sequence, while *lux A* primers should only amplify a 1.1 kbp *lux A* genomic fragment in transgenic plants. In addition, various plasmids were used as positive and negative controls (see Fig. 6).

The precaution of using *PAL1* primers as a positive control proved to be a valuable method for assessing the quality of the DNA template. Some DNA preparations from putative transgenic lines produced no *lux A* product, thus appearing to indicate a

Figure 6: PCR analysis of genomic DNA from kanamycin-resistant, transgenic, *Arabidopsis* plants. The upper picture shows the amplification of the full-length *PAL1* promoter using primers 1 and 2 (from Fig. 2). Lanes 1 and 2 contained pBluescript KS+ and pPAL45 template DNA, as negative and positive controls, respectively. Lanes 3 and 4 contain as template, *Landsberg erecta* and RLD total genomic DNA from nontransformed plants. Lanes 5, 6, 7 and 8 contained total genomic DNA from transgenic lines #2, #3, #5 and #6, respectively, as template. The bottom gel shows the amplification of the *lux A* fragment of the *lux F* reporter gene. Lanes 1 and 2 contain pPAL45 and pGL101 as negative and positive controls, respectively. Lanes 3 and 4 contained genomic DNA from ecotypes *Landsberg erecta* and RLD. Lanes 5, 6, 7 and 8 contained genomic DNA from transgenic lines #2, #3, #5 and #6. A 3 μL aliquot from each 50 μL reaction mixture was visualized on a 1% TBE agarose gel, by EtBr-UV fluorescence. Lane M indicates *Hind*III digested λ DNA which was used as the molecular-weight marker.

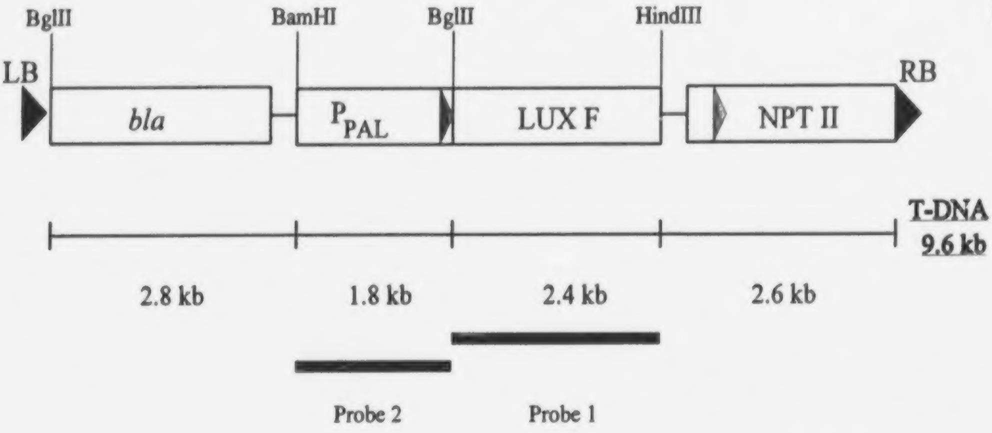

M 1 2 3 4 5 6 7 8 M
PAL1
promoter
1.8 kbp

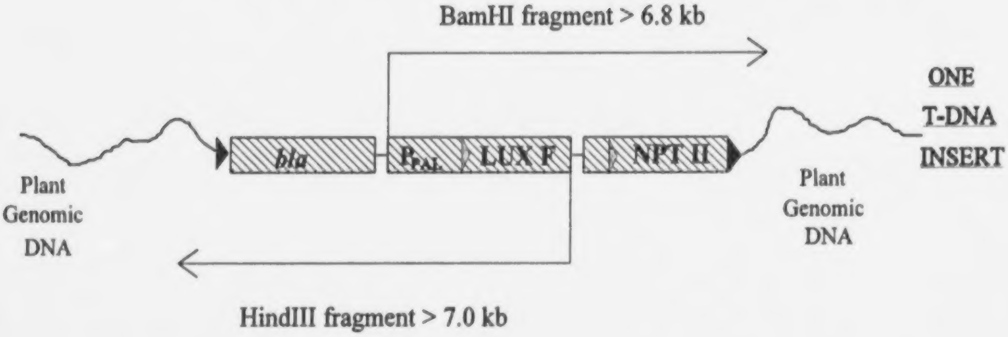

M 1 2 3 4 5 6 7 8 M
lux A
1.1 kbp

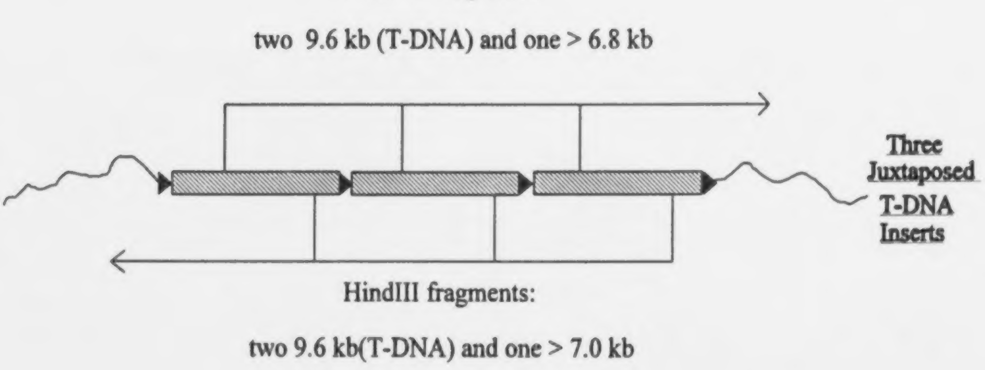
nontransformed plant line. However, when the same samples were analyzed with *PAL1* primers, no *PAL1* promoter PCR product was generated thereby indicating a problem in the overall quality of the DNA template. The rapid "mini-procedure" used to isolate DNA template for PCR often yielded DNA preparations of unreliable quality. Some preparations were not suitably pure either containing contaminating polysaccharides or unidentified inhibitors of *Taq* polymerase. This difficulty was noted previously with DNA preparations isolated from plants (Hamill *et al.*, 1991; Gasch *et al.*, 1992) and two remedies were suggested: i) use CsCl-purified DNA template, or ii) use less template DNA which will decrease the amount contaminants in the reaction mixture. Instead of these solutions, further attempts to isolate genomic DNA were made; these proved successful and demonstrated that indeed the putative transgenic plants do in fact contain both the *lux A* and *PAL1* promoter sequences, while the wild-type genomic DNA from *Arabidopsis* ecotypes RLD and *Landsberg erecta* possessed only *PAL1* promoter sequences.

Further analysis of genomic DNA from these transgenic plants was carried out using Southern hybridization. Genomic DNA in sufficient quantity and quality was difficult to obtain reproducibly using the method of Doyle and Doyle, especially when large amounts of plant tissue were required for high yields of DNA. Consequently, a modified procedure which incorporated an additional CTAB extraction step to remove much of the polysaccharide was employed (II.12.2). This modification resulted in relatively high yields of DNA of sufficient quality that could be readily digested by restriction endonucleases. Also a 1 M NaCl precipitation step was carried out after restriction endonuclease digestion, to remove remaining polysaccharides and thus facilitate loading of small volumes of digested genomic DNA onto agarose gels (Girowei *et al.*, 1992).

Figure 7: The T-DNA region located on pGL101 spanning the left and right border regions with the restriction enzyme sites which are relevant to Southern hybridization analysis. The 9.6 kbp T-DNA fragment from pGL101, containing the *PAL1-lux F* transcriptional fusion, the neomycin phosphotransferase gene (NPT II), the bacterial ampicillin-resistance gene (*bla*) and the two bordering 24 bp direct repeats (LB and RB), is transferred into plant genome upon infection by *A. tumefaciens*. P_{PAL} and Lux F indicate the *PAL1* promoter and the *lux F* reporter gene. The hypothetical structures of a single insert (middle) and of multiple, juxtaposed, tandem inserts (bottom) are shown, with the predicted minimum size of DNA fragments produced by *Bam*HI or *Hind*III restriction enzyme digestion of total genomic DNA from transformed *Arabidopsis* plants. In the case of multiple, tandem, juxtaposed T-DNA insertions (bottom), any restriction endonuclease which cleaves within the T-DNA once will generate several 9.6 kbp fragments.


BglII
BamHI
BglII
HindIII
LB
bla
P_PAL
LUX F
NPT II
RB
T-DNA
9.6 kb
2.8 kb
1.8 kb
2.4 kb
2.6 kb
Probe 2
Probe 1


BamHI fragment > 6.8 kb
ONE
T-DNA
INSERT
bla
P_PAL
LUX F
NPT II
Plant
Genomic
DNA
Plant
Genomic
DNA
HindIII fragment > 7.0 kb


BamHI fragments:
two 9.6 kb (T-DNA) and one > 6.8 kb
Three
Juxtaposed
T-DNA
Inserts
HindIII fragments:
two 9.6 kb(T-DNA) and one > 7.0 kb

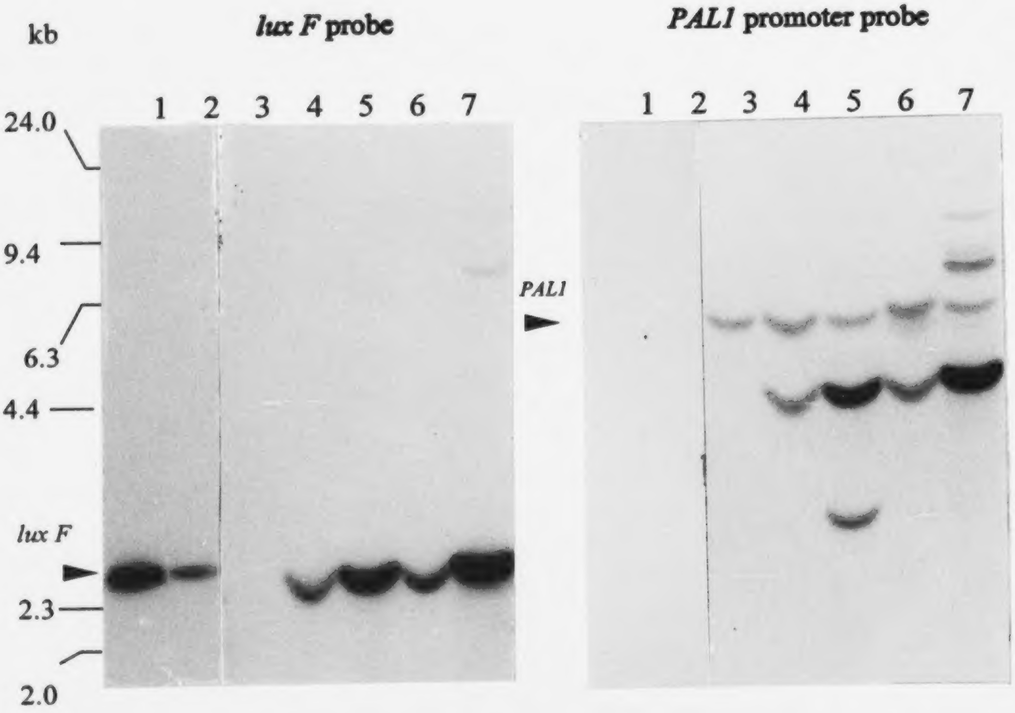
Initially a "double-digest" using the restriction enzymes *Hind*III and *Bgl*II, which would excise the 2.4 kbp *lux F* reporter gene from the T-DNA (containing the poly-A site and 23 nucleotides of the *PAL1* promoter), was employed (see Fig.7). The 2.4 kbp *lux F* fragment was excised from plasmid pGL101 and 200 pg and 50 pg were loaded onto the agarose gel as a positive control. The genomic DNA fragments were separated on a 1% TAE agarose gel, were blotted on a nylon membrane and were hybridized to a ^{32}P radio-labelled probe generated from 100 ng of the same *Hind*III-*Bgl*II *lux F* fragment referred to above. This analysis, shown in Figure 8, revealed that plants from the transgenic plant lines #2, #3, #5 and #6 possess the *Hind*III-*Bgl*II *lux F* reporter gene fragment, while the wild-type genomic DNA showed no genomic fragments hybridizing, whatsoever, to the *lux F* probe. The strength of the signals from #2 and #5 were similar, while the strength of the hybridization signals from #3 and #6 were more intense indicating that #2 and #5 likely possess a single insert each, while #3 and especially #6 possess multiple inserts.

This *Hind*III-*Bgl*II genomic blot was further analyzed by removing the *lux F* probe and rehybridizing the membrane to a radioloabelled probe generated from the 1.8 kbp *Bam*HI-*Sal*I *PAL1* promoter from pPAL45. Genomic DNA from nontransformed, wild-type, *Arabidopsis* plants served as a positive control and revealed a 6.0 kbp *Bgl*II-*Hind*III genomic fragment which contained the endogenous *PAL1* promoter; this genomic fragment was common to all the genomic samples and was of similar intensity in all samples. A 4.6 kbp *Bgl*II fragment from pGL101 was present in only the genomic samples from the transgenic plants providing further proof that the kanamycin-resistant lines are in fact transgenic plants and do possess foreign DNA containing the *lux F* reporter gene fused to the full-length *Arabidopsis PAL1* promoter as constructed in pGL101. Again, the strength of the 4.6 kbp *Bgl*II bands in #2 and #5 were approximately equal, while #3 and #6 possessed bands of much stronger intensity indicating the presence of multiple copies of inserts. In addition, a fast migrating DNA fragment, which strongly hybridized to the *PAL1* probe, was observed in genomic digest from transgenic line #3, revealing that in one instance a deletion in one T-DNA may have occurred during the integration process. The hybridization signal was of equal intensity to the single resident *PAL1* promoter bands reported above and migrated at approximately 2.5 kbp indicating that the deletion may have occurred at the left border terminus, probably in the ampicillin-resistance gene rather than in the *PAL1* promoter itself.

Detailed analysis of individual T-DNA insertions has previously shown that T-DNA is subject to rearrangements especially in the region of T-DNA-plant DNA junctions near the right and left borders (Koncz *et al.*, 1992). Specifically, the examination of left T-DNA border regions indicate that complete T-DNA copies are not always transferred into the genome. In fact, in one recent report, both leaf tissue and root-derived transgenic plants, frequently (63% and 46%, respectively) did not contain about 3.0 kbp of the left border-linked region (Grevelding *et al.*, 1993). It is crucial to determine how much of the remaining T-DNA was transferred into the genome. Based upon the strength of the hybridization signal and size of the hybridizing band it appears as though in one instance (transformant #3) the full-length *PAL1* promoter was indeed transferred, although a nearly 2.0 kbp deletion may have occurred in the left border region of the T-DNA, probably within the *bla* gene.

To determine the number of T-DNA insertion into the plant genome and further characterize the genotypes of the 4 transgenic lines, *Arabidopsis* genomic DNA was further analyzed by digestion with a single restriction endonuclease which cleaves within the T-DNA insert at only one site. Any fragment released from the endonuclease digestion would therefore contain most of the foreign T-DNA and a portion of plant genomic DNA associated with it (see Fig. 7).

Figure 8: Southern hybridization analysis of total genomic DNA isolated from transgenic *Arabidopsis* plants after "double-digestion" with *Hind*III and *Bgl*II. The total genomic DNA was isolated from 3-4 week-old *Arabidopsis* plants and 5 μg was digested in a "double-digest" with *Hind*III and *Bgl*II. The DNA fragments were separated on an agarose gel, and blotted and fixed onto a nylon membrane. The immobilized DNA fragments were then analyzed by hybridization to a radiolabelled *lux F* probe. After exposure to film, the *lux F* probe was "stripped-off" the membrane and the DNA fragments were rehybridized to a radiolabelled *PAL1* promoter probe. Lanes 1 and 2 contain 200 pg and 50 pg, respectively, of the 2.4 kbp HindIII-*Bgl*II *lux F* fragment from pGL101 as the positive control. Lane 3 contains genomic DNA from nontransformed plants, while lanes 4, 5, 6 and 7 contain genomic DNA from transgenic lines #2, #3, #5 and #6, respectively. *Hind*III digested λ DNA was used as the molecular weight marker. The arrows indicate the 2.4 kbp *lux F* reporter gene fragment (left) and the endogenous, 6.0 kbp, *PAL1* promoter-containing fragment (right).


kb
lux F probe
PAL1 promoter probe
1 2 3 4 5 6 7
1 2 3 4 5 6 7
24.0
9.4
6.3
4.4
2.3
2.0
lux F
PAL1

Genomic DNA was first digested with *Bam*HI and the DNA fragments were separated on an agarose gel and blotted onto a nylon membrane as above. The blot was hybridized first with a *lux F* probe. The *lux F* probe was removed and the blot was reprobed with a *PAL1* promoter probe as performed previously. This analysis revealed that the transgenic plant lines #2 and #5 do in fact possess only one insert each, while #3 showed three hybridizing bands indicating that it possessed three inserts (see Fig. 9). Genomic DNA from transgenic line #6 revealed the presence of an intensely hybridizing 9.6 kbp band which corresponds exactly to the full length T-DNA insert, in addition to several other hybridizing bands. The strongly hybridizing band of 9.6 kbp suggests the presence of multiple, adjoining, tandem insertions while several distinct migrating bands indicate separate or uncoupled insertions. The hybridization with the *PAL1* probe revealed a single 5.0 kbp *Bam*HI DNA fragment containing the endogenous *PAL1* promoter common to wild-type and transgenic genomic samples, as well as the additional bands detected above only in the genomic samples from transgenic plants. The genomic fragments (containing the foreign T-DNA) which hybridized above to the *lux F* probe, also hybridized to the *PAL1* probe thereby demonstrating that the *PAL1* promoter is in fact fused to the *lux F* reporter gene.

Genomic DNA was also digested with *Hind*III and the genomic fragments were hybridized to the same *lux F* and *PAL1* radiolabelled probes as discussed above (see Fig. 10). This analysis verified that transgenic lines #2 and #5 possessed only one insert, while #3 possessed three inserts. Again, #6 showed an intense 9.6 kbp fragment thus providing additional proof that this transgenic line possesses multiple tandem insertions, in addition to separate insertions. The hybridization with the *PAL1* probe revealed a single 9.3 kbp band containing the endogenous *PAL1* promoter sequence common to nontransformed as well as transformed plants, in addition to the extra bands detected by

Figure 9: Southern hybridization analysis of genomic DNA isolated from transgenic *Arabidopsis* plants and digested with *Bam*HI. Total genomic DNA was digested with *Bam*HI, seperated by electrophoresis through an agarose gel and blotted onto a nylon membrane as in Figure 8. The contents of the lanes correspond exactly to the contents of the lanes in Figure 8. The nylon membrane was first probed with a *lux F* probe; this probe was "stripped-off" and the membrane was reprobed with a *PAL1* promoter probe as in the previous Figure 8. The arrows indicate the 2.4 kbp *lux F* fragment (left) and the endogenous, 5.0 kbp, *PAL1* promoter-containing fragment (right).

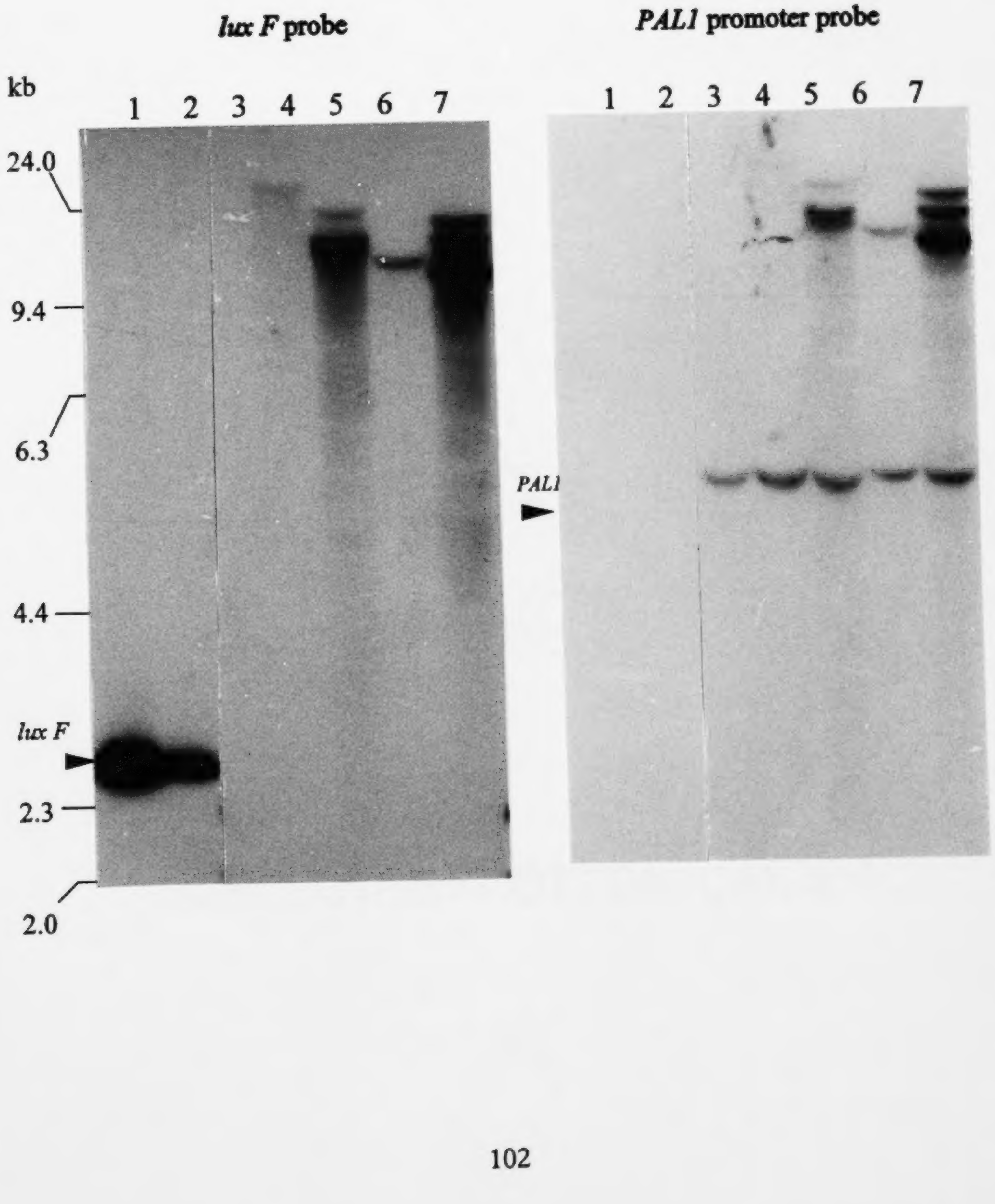
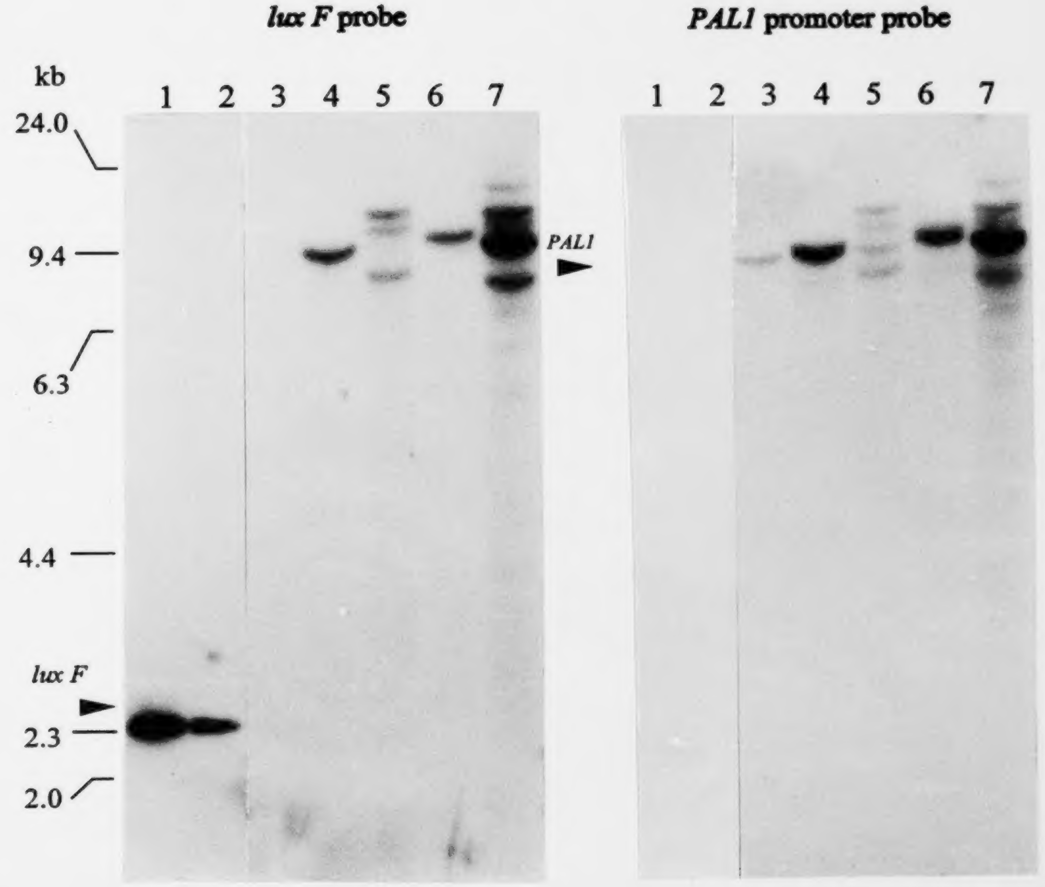

lux F probe
PAL1 promoter probe
kb
1 2 3 4 5 6 7
24.0
9.4
6.3
4.4
lux F
2.3
2.0
1 2 3 4 5 6 7
PAL1

Figure 10: Southern hybridization analysis of genomic DNA isolated from transgenic *Arabidopsis* plants and digested with *Hind*III. The contents of the lanes in this Figure corresponds exactly with the previous Figures 8 and 9. The arrows indicate the 2.4 kbp *lux F* fragment (left) and the endogenous, 9.4 kbp, *PAL1* promoter-containing genomic fragment (right).


lux F probe
PAL1 promoter probe
kb
1
2
3
4
5
6
7
24.0
9.4
6.3
4.4
lux F
2.3
2.0
PAL1
1
2
3
4
5
6
7

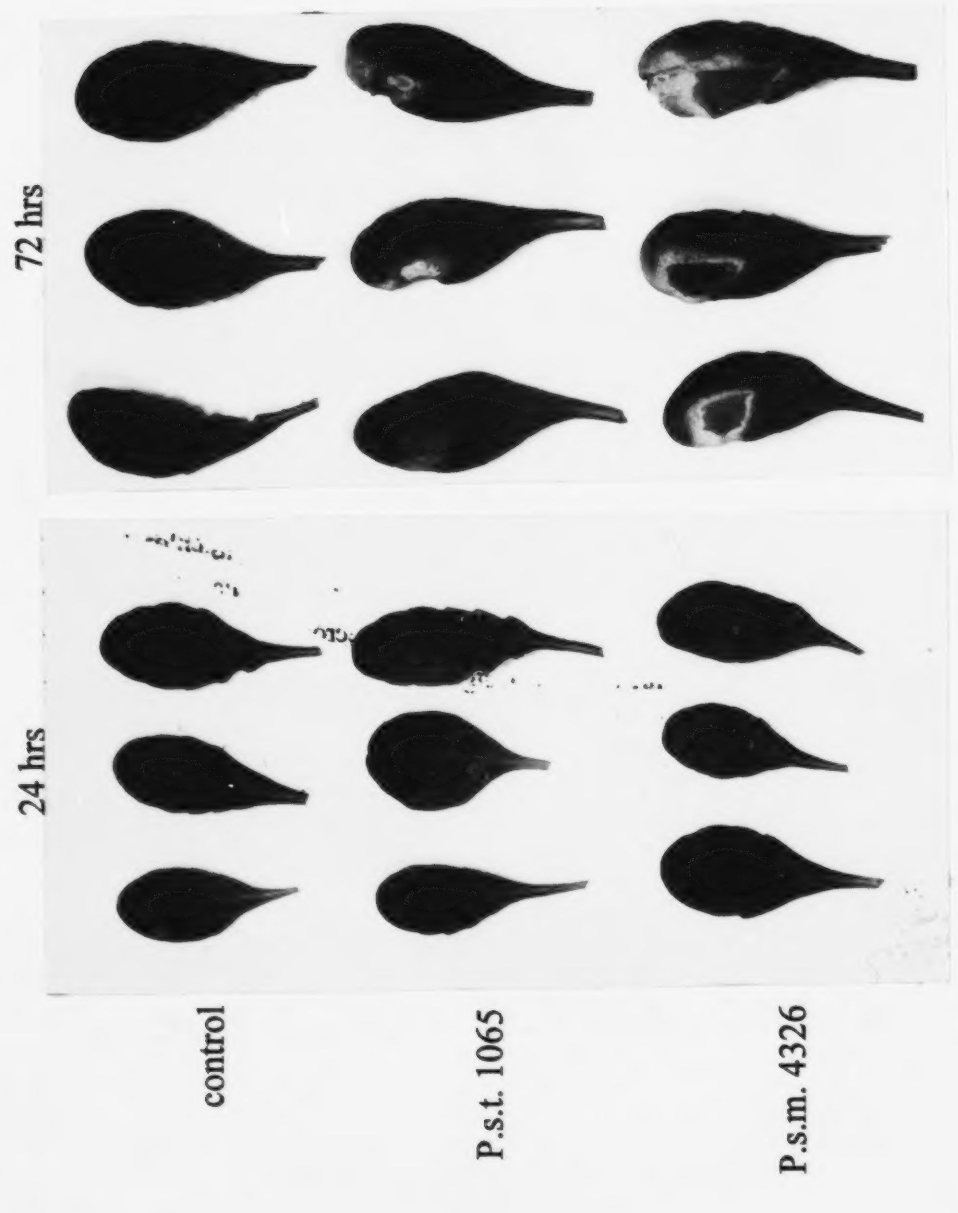
hybridization with the *lux F* probe, thus proving unequivocally that #2, #3, #5 and #6 are all individual transformants and that both #2 and #5 possess single inserts each, while #3 possesses 3 inserts (one of which may have a deletion in the left border region) and # 6 possesses multiple inserts including several, tandem, juxtaposed repeats. Multiple T-DNA insertions have been commonly observed and are frequently organized as direct "head-to-tail" or "head-to-head" arrays (Grevelding *et al.*, 1993).

Altogether, the analysis of the segregation of kanamycin-resistance and Southern hybridizations of genomic DNA from transgenic plants correspond to previous information on *Agrobacterium*-mediated transformation of *Arabidopsis*, which asserts that multiple T-DNA inserts are common, although they usually occur at only 1 or 2 loci and that dimeric or trimeric juxtaposed T-DNA repeats do occur. No evidence of rearrangements within the T-DNA inserts was detected.

III.6 MONITORING PATHOGEN-INDUCED LUCIFERASE ACTIVITY *IN VITRO*, USING AN ENZYMATIC ASSAY

The inoculation of *P. syringae* pv. *tomato* (*Pst*) and pv. *maculicola* (*Psm*) into *Arabidopsis* leaves resulted in well-known disease symptoms already reported in the literature (Debner *et al.*, 1991; Dong *et al.*, 1991; Whalen *et al.*, 1991). Typically, leaves infiltrated with either *Pst* or *Psm* exhibited visible, dry, light-brown lesions at the infiltration site within 24 hrs after infiltration; these were for the most part indistinguishable. However, 2 to 3 days after infiltration, the *Pst* infiltrated leaves showed only a little spreading of the necrotic, dry, papery lesion and in some instances demonstrated very weak chlorosis. In contrast, strong disease symptoms characterized by a brown necrosis surrounded by a spreading, water-soaked, chlorotic halo were typically observed in leaves infiltrated with *Psm* (see Fig. 11). Five to seven days after infiltration with *Pst* leaves still remained viable, although some minor spreading of the dry necrotic

Figure 11: The development of contrasting disease symptoms in *Arabidopsis* leaves infiltrated with avirulent and virulent *P. syringae* strains. The middle left-edge of one leaf was infiltrated with 5 µL of a bacterial suspension as described in Materials and Methods (II.13.1). After 24 hrs, there was no significant differences in disease symptoms between the two strains; a visible dry necrotic lesion was observed at the site of infiltration in most cases. After 48 hrs the leaves infiltrated with Psm 4326 began to show the formation of chlorotic haloes around the lesion (not shown). After 72 hrs, a significant difference was observed between the leaves infiltrated with Pst 1065 and Psm 4326. Lesions generated by *Psm* infection began to expand and showed large, spreading, chlorotic haloes surrounding the centrally-located, dry, brown zones. This enlarging chlorotic region was characteristically water-soaked and eventually spread to engulf the whole leaf. Lesions caused by *Pst* were characterized by a dry light-brown necrosis, which was sometimes only very weakly chlorotic and which expanded significantly slower than the lesions caused by *Psm*.


24 hrs
72 hrs
control
P.s.t. 1065
P.s.m. 4326

lesion was observed. In contrast, 5-7 days after infiltration with *Psm*, most leaves were completely water-soaked, drooping and chlorotic over the whole leaf surface indicating unrestricted bacterial colonization of the leaf, concluding in leaf collapse. Thus, the two isogenic *P. syringae* strains confer two contrasting disease symptoms on *Arabidopsis* plants: *Pst* induces a characteristic HR and is avirulent, while *Psm* causes severe disease symptoms and is therefore virulent.

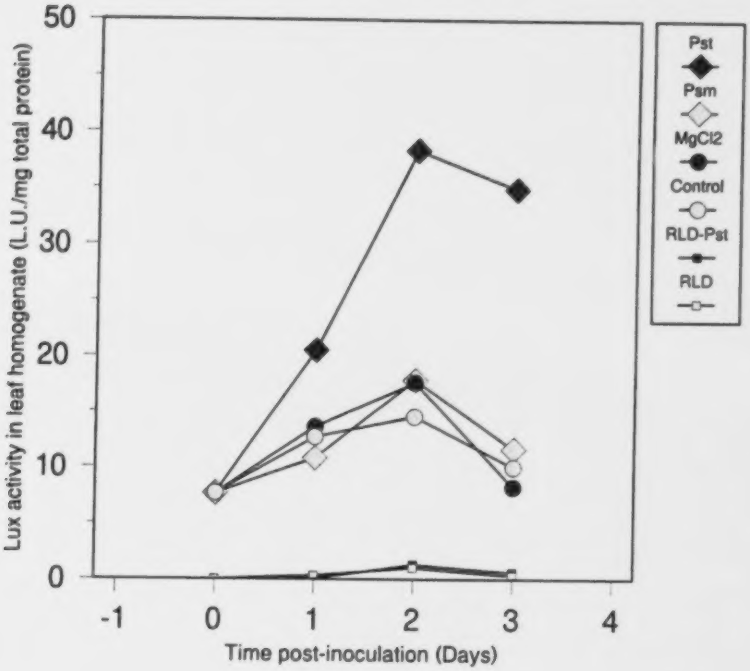
For measuring bacterial induced *PAL1* promoter-*lux F* reporter gene expression an *in vitro* luciferase assay was used (Langridge *et al.*, 1989). The same homozygous transgenic plant lines which were characterized by Southern hybridization, #2, #3, #5 and #6, were chosen. One plant leaf per transgenic plant was infiltrated with either *Pst* or *Psm*. To assess the possible wound-effects of the infiltration procedure, a "mock-inoculation" with a solution of sterile $MgCl_2$ (10mM) was carried out. As a control to determine the basal levels of luciferase activity in healthy transgenic plant leaf tissue, some plants were left uninfiltrated and a minimum of three similarly healthy leaves which were chosen for infiltration studies were removed and were assayed directly in the luminometer as uninfiltrated controls.

Numerous microbes are bioluminescent and levels of increased luminescence from transgenic leaves could be caused by the *Pseudomonas* bacteria. Therefore, to test for bioluminescence in the avirulent *P. syringae* strain, leaves from nontransgenic *Arabidopsis* plants were infiltrated with the avirulent *Pst* bacteria, while some leaves were left untreated as the uninoculated control. The luciferase assay was performed at the same time and in exactly the same way for both transformed and nontransformed leaf samples.

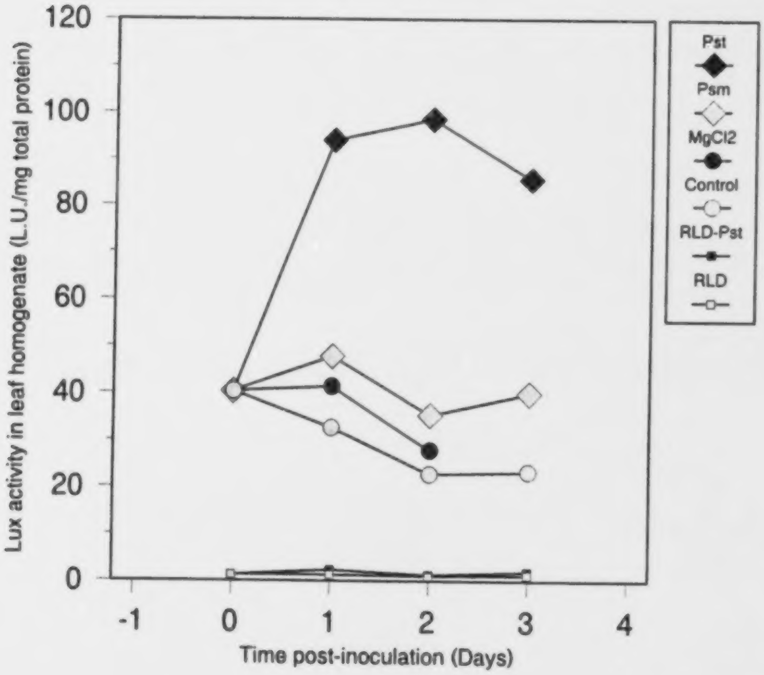
The results of the luciferase assays from bacteria infiltrated leaves, taken from four independent transgenic *Arabidopsis* lines, are presented in Figure 12. The results demonstrate several important points. First, there was no difference in the luciferase measurements from uninfiltrated, control (nontransgenic) *Arabidopsis* leaves and leaves infiltrated with *Pst*, therefore, the avirulent *P. syringae* bacteria are not a source of luminescence in these assays. Second, all the luciferase measurements from the leaves of transgenic plants, with the exception of leaves from transgenic line #6, were of an order of magnitude greater than the luciferase measurements from the nontransgenic counterparts, thus, the background luminescence in these assays is essentially zero. Third, this analysis demonstrates that the *lux F* reporter gene is in fact present and functioning in transgenic plants.

With respect to *PAL1* activation, in all four transgenic lines an increase in luciferase activity of about 3-fold was noted in transgenic *Arabidopsis* leaves, usually within 24 hrs after infiltration with *P. syringae* pv. *tomato*. In contrast, only slight increases in the luciferase activities were noted in those leaves infiltrated with the virulent *Psm* strain or "mock-inoculated" with $MgCl_2$. Number 5 transgenic plants displayed a somewhat slower induction of *PAL1-lux F* expression which occurred between 48 and 72 hrs after infiltration. This specific deviation and any other minor differences were attributed to random variations in the general physiological state of the plants. Slight differences in the age of the plants, soil, humidity, light intensity, photoperiod and bacterial inoculations are only a few of the many subtle factors which could effect *PAL1* promoter induction in individual plant leaves. It should be noted that the plants used in this study are the T_2-progeny of a single transgenic T_1-plant, although they share a common genetic background, the T_2-plants are siblings and are not clones, thus, there does exist a certain inherent amount of genetic variation between individual plants which could contribute to subtle variations between samples. In entirety (see Fig. 13), these data show some correspondence to a previous report which documented an average 8-fold

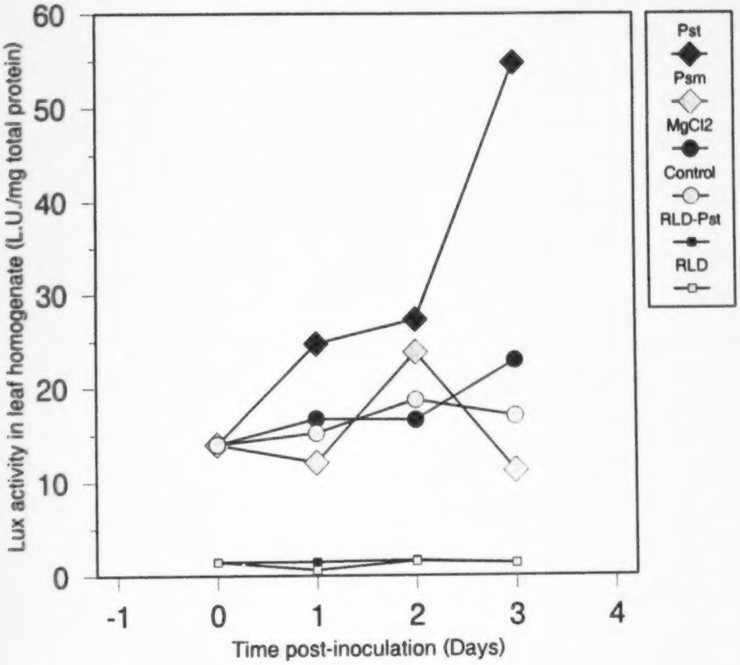
Figure 12: The kinetics of induction of *PAL1-lux F* expression in *Arabidopsis* leaves after inoculation with avirulent and virulent strains of *P. syringae*. These data were collected from the 4 independent transgenic plant lines as indicated. The middle left-edge of leaves were infiltrated with avirulent (Pst) and virulent (Psm) *Pseudomonas syringae* strains as in Figure 11. After infiltration *Arabidopsis* leaves were removed and assayed by luminometry. Each time point represents the average luciferase activities from a minimum of 3 leaves; MgCl2 represents leaves "mock-inoculated" with 10 mM $MgCl_2$; Control represents untreated healthy leaves; RLD represents untreated healthy leaves taken from nontransgenic *Arabidopsis* plants; and RLD-Pst represents leaves from nontransgenic *Arabidopsis* plants infiltrated with *Pst*. The average standard deviation for each data point from transgenic plants #2, #3 and #5 is 36%. Results from transgenic plants #6 represent averages of 4-7 leaf samples since large, but regular variations were observed between samples, thus more leaf samples were examined in an attempt to investigate the variations (continued on page 112).



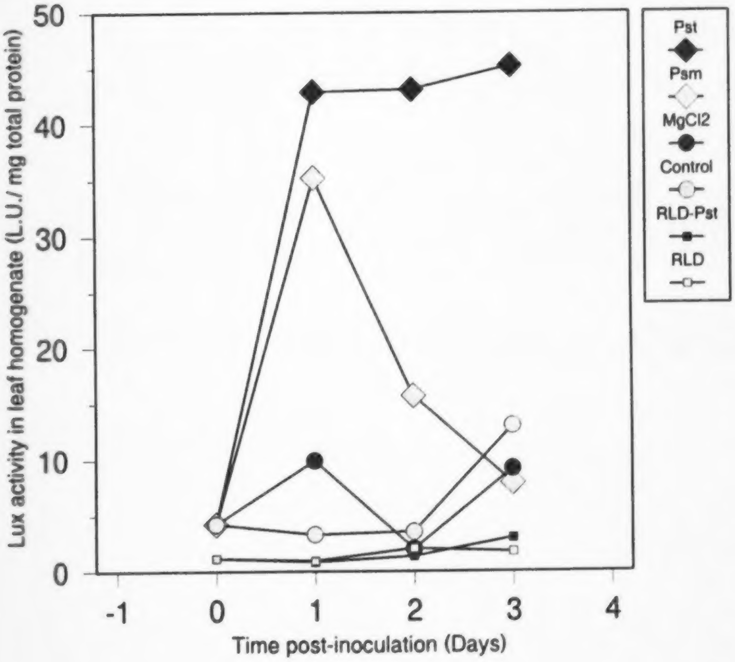
Transgenic line #2



Transgenic line #3



Transgenic line #5



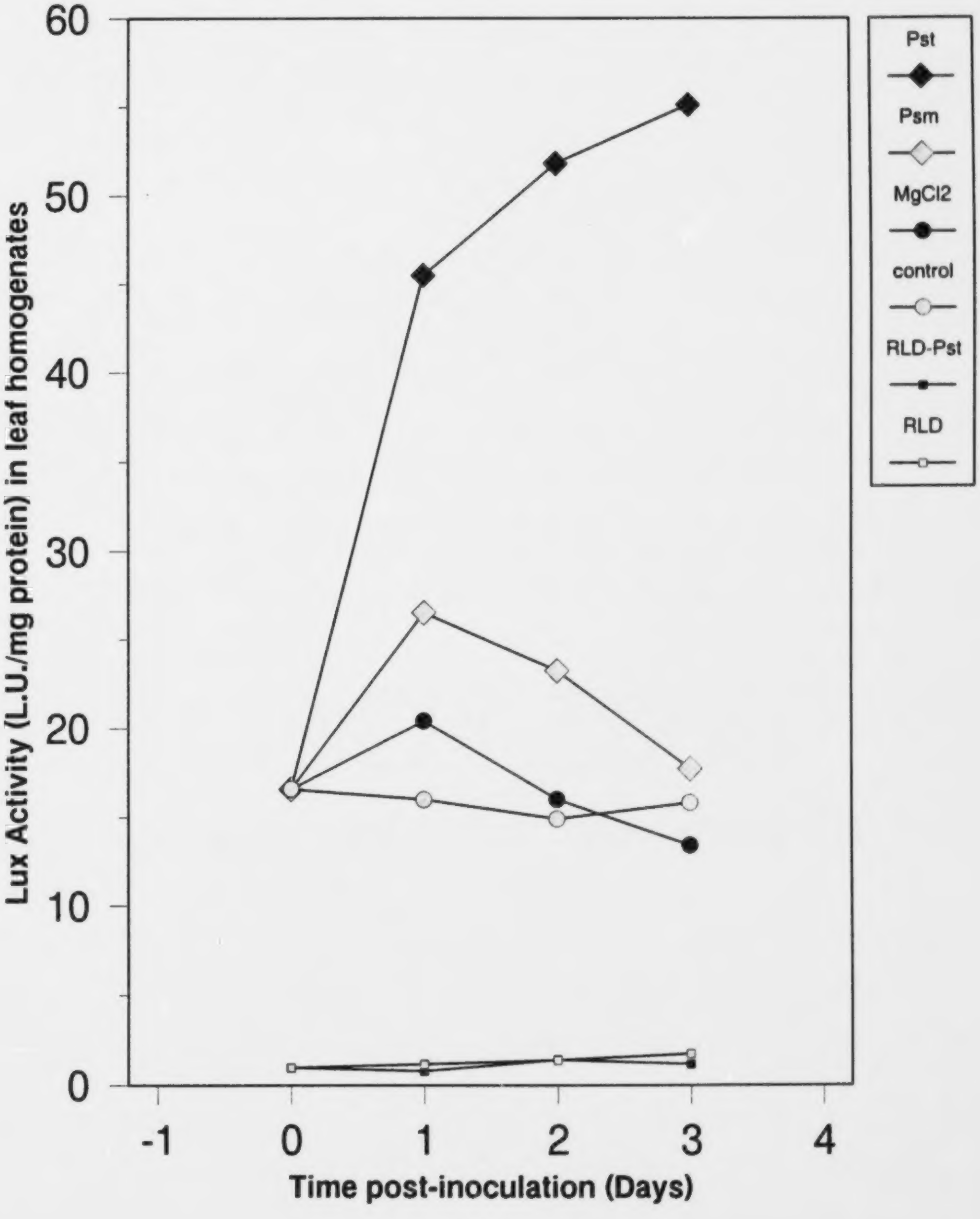
Transgenic line #6

increase in PAL mRNA, observed in *Arabidopsis* leaves (ecotype Columbia), 8 hrs after infiltration with the same avirulent *P. syringae* pv. *tomato* strain, in comparison to leaves infiltrated with the virulent *P. syringae* pv. *maculicola* strain (Dong *et al.*, 1991). The 4-5-fold increases in luciferase activity observed in *Arabidopsis* leaves infiltrated with the avirulent bacteria also generally remained stable 2-3 days after infiltration. Whereas the slight increases in luciferase activities observed in those leaves infiltrated with the virulent bacteria, or "mock-inoculated" with $MgCl_2$, decreased after 24-48 hrs to basal levels, although the kinetics of induction in all three treatments are identical. These results demonstrate that the avirulent *P. syringae* pv. *tomato* bacteria are able to elicit the induction of a specific defense reaction, the activation of the *PAL1* promoter, while the virulent bacteria, *P. syringae* pv. *maculicola*, elicit only minor increases in *PAL1* promoter activity and overall the luciferase expression remained largely unaffected.

Among specific transgenic lines there also exists a correlation in the luciferase activities (see Fig. 12) and the previous data from Southern hybridization analysis. The basal levels of luciferase activities observed in the leaves from transgenic lines #2 and #5 which contain only one transgene copy each were found to be relatively comparable and calculated to be 11.2 and 16.2 L.U./mg total protein, respectively. While transgenic line #3 expressed basal levels of luciferase activity of 34.2 L.U./mg protein, 2.5-times the average luciferase activity of 13.7 L.U./mg protein, found in transformed lines #2 and #5. Accordingly, transgenic line #3 possesses 3 copies of the *PAL1* promoter-*lux F* reporter gene construct. Thus these data show a direct correlation between increased transgene copy-number and increased reporter gene activity.

Southern hybridization analysis of DNA isolated from transgenic line #6 indicates that it contains multiple, tandem, juxtaposed inserts of foreign DNA. In general, this transformant yielded extremely low levels of luciferase activity which for the most part

Figure 13: The average kinetics of induction of *PAL1-lux F* expression in *Arabidopsis* leaves after inoculation with avirulent and virulent *P. syringae* strains. Data from Fig. 12 were averaged to determine the overall kinetics of induction of the *PAL1-lux F* construct in 4 individual lines of transgenic *A. thaliana*. The labelling of the graph corresponds exactly to that in Fig. 12.

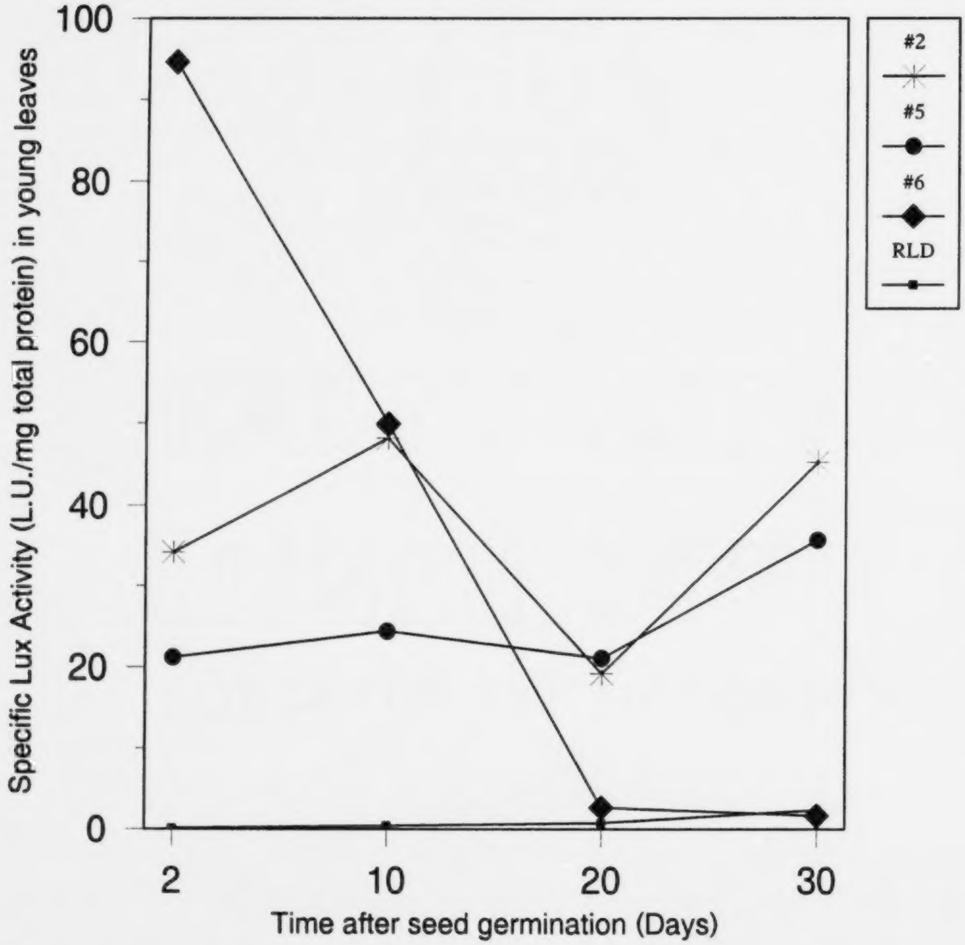


were indistinguishable from the nontransgenic control plants. Only one plant in four showed measurable levels of reporter gene activity; these were slightly lower than those observed in transgenic line #3. The introduction of a transgene into the plant genome does not always result in optimal levels of expression of the introduced gene due to "co-suppression" or "gene-silencing", thus the number of transgene copies in plants may not always be directly related to gene-expression levels. (Jorgensen, 1990). This phenomenon has also been recently attributed to the structure of the introduced transgene, within the plant genome. Insertion of two identical genes which were juxtaposed in a head-to-tail inverted repeat manner resulted in gene-silencing. Interestingly, epigenetic gene-silencing was found to be reversible and was correlated with a decrease in steady-state mRNA and increased DNA methylation. In addition, other transgene copies located at distinct unlinked loci appeared to be silenced. However, Mendelian-segregation of the single transgene from the inverted repeat structure, in some of the progeny, resulted in the reactivation of the transgene (Assad *et al.*, 1993; Hobbs *et al.*, 1993). The mechanism of action of this phenomenon is not yet understood, although previous findings suggest that the gene-silencing reflects interactions of paired homologous DNA strands, with each other, which induces methylation and condensation of chromatin into a nontranscribable state.

Southern hybridization results of genomic DNA from #6 transgenic plants also revealed some high molecular-weight, weakly-hybridizing, DNA fragments. These partially digested genomic fragments were specific to the transgene fragments, since there were no differences seen on the EtBr-stained agarose gel, in the banding patterns between any of the DNA samples. This raises the possibility that specific methylation of segments of genomic DNA containing the transgene may have occurred. Specific methylation of transgenes has been shown previously and is characteristic of gene-silencing. I believe that transgenic line #6, which possesses multiple tandem insertions of T-DNA, is generally showing low levels of luciferase activity due to epigenetic gene-silencing or co-suppression, phenomena which have been previously observed in transgenic plants. The #6 T_2-plant progeny which were periodically detected at a frequency of 1/4 to be exhibiting high levels of reporter gene activity, are in fact progeny which have an a active reporter gene (or reporter genes) at one locus which has segregated from the other locus which contains the juxtaposed T-DNA insertions implicated in silencing gene expression (transgenic line #6 contained two kanamycin-resistance loci). The #6 T_2-plants chosen in this study may be homozygous for a single "luciferase expressing locus", but are heterozygous for a head-to-tail "silencing locus" containing multiple juxtaposed inserts. When segregation occurs during meiosis and gamete formation, the segregated locus containing the "expressing locus" may be able to reactivate in the developing embryo and later in the plant. Another possible explanation for the low levels of luciferase activity found in this transformant may be that the high copy-number of transgenes are interfering with the basal and even microbial-induced expression of the promoter by titration of *PAL1* promoter-specific transcription factors out of the pool of transcription factors required for normal promoter activity.

To further examine the gene-silencing phenomenon a developmental study was initiated. Leaves emerging from the top of the rosette, from seedlings at various stages of growth, were removed and assayed quantitatively for specific luciferase activity using the luminometer. *Arabidopsis* seeds from transgenic lines #2, #5 and #6 were plated on MS 1.25% supplemented with kanamycin. Cotyledons from seedlings approximately 2 days post-germination were assayed first, then for the 10, 20 and 30 day samples, young leaves (1-1½ weeks old) which had recently emerged from the top of the rosette and were of approximately the same size were assayed. As depicted in Figure 14, the specific

Figure 14: Changes in *PAL1-lux F* expression during plant growth and development. Leaves were obtained from three independent transgenic *Arabidopsis* lines. One and a half to two week-old leaves, which had recently emerged from the crown of the rossete, were removed and assayed for luciferase activity (L.U./mg total protein) using a luminometer. The 3 day time point of luciferase activity was taken from recently emerged cotyledons of seedlings. RLD represents the nontransformed *Arabidopsis* leaves



luciferase activities in leaves from plants from transgenic lines #2 and #5 showed an overall stable pattern of expression throughout the 30 day interval, with slight variations being largely dependent on minor differences in leaf-size and stage of growth. In these transgenic plants, young plant tissue emerging from the crown of the rosette, regardless of the age of the plant, displayed similar levels of luciferase activity. The luciferase activities in the leaves of these two lines was roughly equivalent which is indicative of the fact that transgenic plants from both lines possess only one T-DNA insert as was determined by Southern hybridization analysis.

The luciferase activities in leaves from transgenic plants #6 showed a significantly different pattern. The maximum specific luciferase activities were found in the cotyledons taken from three day-old seedlings and was approximately 3-4 fold higher than the luciferase activity in cotyledons taken from transgenic plants #2 and #5. This high level of specific luciferase activity observed at this point was surprising, since from the previous experiment, extremely low levels of luciferase activities were observed in general from the leaves taken from 4-5 week-old #6 plants. Also of significance was the substantial linear decrease in the specific luciferase activity, in leaves taken from transgenic plant line #6, observed as the plant matured over time. This decrease indicates that the *PAL1-lux F* construct is functioning in young plants and that the occurrence of gene-silencing is related to the development and age of the plant and is not related to the age of the specific leaf tissues. This experiment demonstrates that epigenetic gene-silencing may be a dynamic process which is influenced by the growth and maturation of the plant.

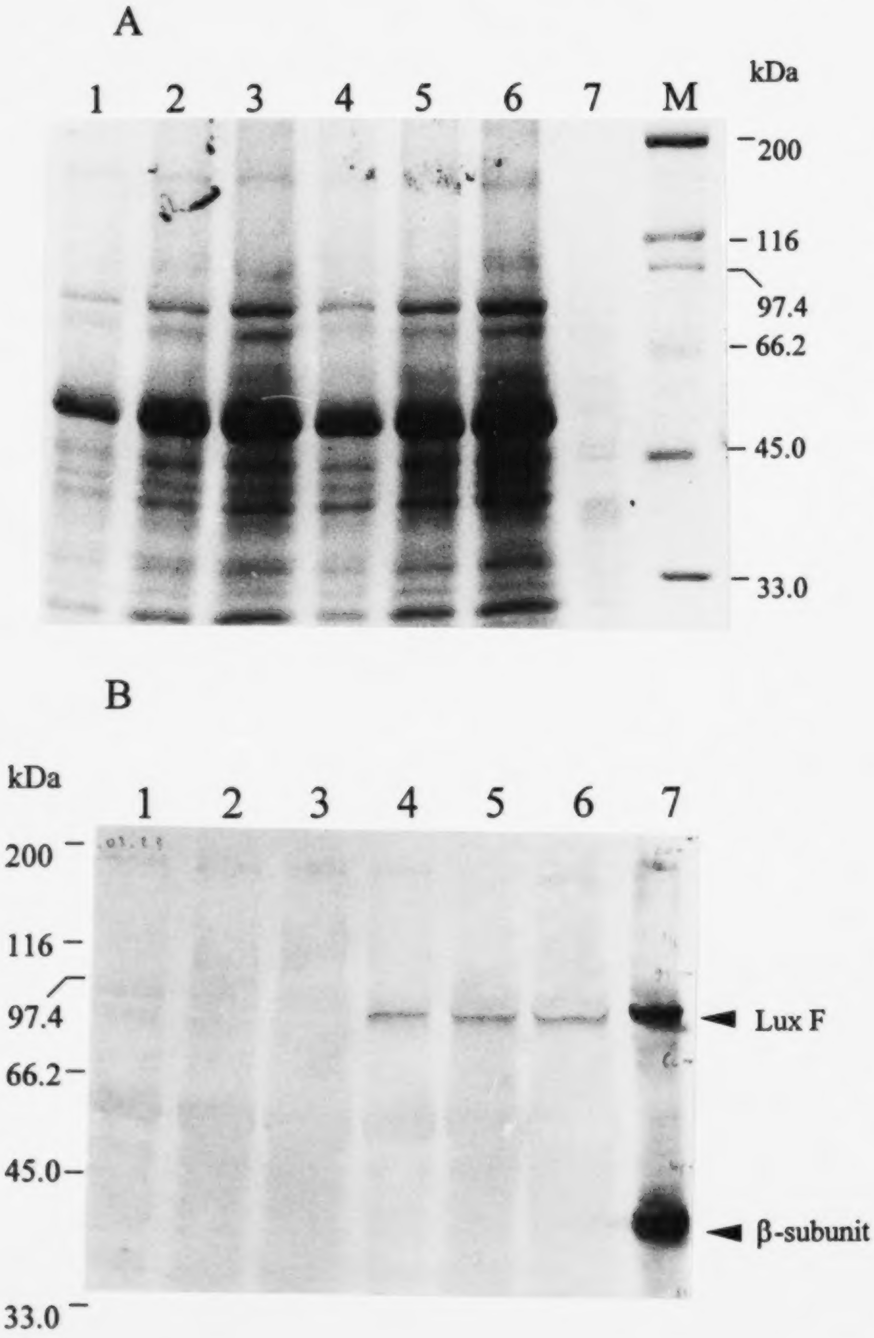
In general these experiments provided quantitative measurements of differences in the reporter gene activity which can be used comparatively for measuring increases in the specific luciferase activity in different *Arabidopsis* leaves. These results demonstrated large specific increases in the luciferase activity of plant leaves inoculated with avirulent *P. syringae* strains. However, two drawbacks of assaying *PAL1-lux F* induction in this manner are: i) the gene expression in specific areas of a leaf cannot be assessed and, ii) changes in one specific leaf cannot be monitored *in vivo*, over time, since the leaf is removed from the plant and is destroyed in the analysis.

III.7 WESTERN TRANSFER AND IMMUNODETECTION OF LUCIFERASE PROTEIN

During experiments to monitor the microbial induction of *PAL1-lux F* expression *in plant*, using plants from transgenic line #3, a few plants began to form an inflorescence (flowering stem). The inflorescence exhibited extremely high levels of luminescence which were not evenly distributed over the whole inflorescence. Quantitative measurements were made of the base, middle and upper portions of the inflorescence using the luciferase assay. These measurements revealed that the segments of the inflorescence base, directly adjacent to the rosette and including the area 1-2 cm above the rosette, possessed the highest levels of luminescence. The average specific luciferase activity obtained from three separate plants was 4420 L.U./ mg protein. This high level of luciferase activity is 47-fold higher than the average levels of bacteria-induced luciferase activity of 92.7 L.U./mg protein (Fig. 12), observed in leaves inoculated with the avirulent bacteria, and is 129 times higher than basal luciferase activity of 34.2 L.U./mg protein recorded in 4-5 week-old, uninfected, leaf tissue. This finding proves that the *PAL1* promoter is regulated in an organ and tissue-specific fashion. The high levels of luciferase activity found in this tissue suggest that this tissue contains high amounts of the luciferase protein which would probably be detected by immunobloting analysis using specific antibodies raised against the Lux F protein.

Inflorescence segments (approximately 2.5 cm in length) taken from the base of

Figure 15: SDS-polyacrylamide gel (A) and immunoblotting analysis (B) of the total proteins prepared from stem tissue from a transgenic *Arabidopsis* line. Lanes 1, 2, and 3 contain 70 μg, 140 μg and 240 μg of total protein from tissue from nontransformed *Arabidopsis* plants. Lanes 4, 5 and 6 contain the same amount of total protein as 1, 2 and 3, prepared from *Arabidopsis* transformed plants (transgenic line #3). Lane 7 contains the total proteins prepared from the lysate of *E. coli* cells overproducing the Lux F protein as positive control. The arrows indicate the Lux F protein (78 kDa) and the β subunit of Lux F fusion protein. Lane M contains 20 μL of Bio-Rad broad range molecular markers which includes the following proteins: Myosin rabbit skeletal muscle (200 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle Phosphorylase-β (97.4 kDa), bovine serum Albumin (66.2 kDa), Ovalbumin (45 kDa) and bovine Carbonic Anhydrase (31.0 kDa). Lane M is not shown in B.


A
1
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M
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200
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97.4
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B
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3
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5
6
7
200
116
97.4
66.2
45.0
33.0
Lux F
β-subunit

the inflorescence, adjacent to the rosette, were prepared by grinding in a mortar and pestle. This tissue was extremely dry, difficult to homogenize and displayed an almost "woody-type" texture, quite unlike other vegetative plant tissue. Three different amounts of protein samples (70 μg, 140 μg and 240 μg) were loaded onto an SDS-PAGE, since the minimum sensitivity of immunoblotting protocols to the detection of the Lux F protein in homogenates was not known (see Fig. 15). A negative control was prepared from the same tissue taken from nontransformed *Arabidopsis* plants and a positive control was prepared from *E. coli* overproducing the Lux F protein, were also loaded on the same gel. The gel was electrophoresed overnight and the separated proteins were electroblotted onto a PVDF membrane. Immunodetection was employed to detect the presence of the Lux F protein on the western blot. The blot revealed the presence of the 78 kDa Lux F protein from the positive control. The 78 kDa protein found in the total protein samples prepared from #3 plants migrated exactly at the same molecular weight as the Lux F protein from the *E. coli* positive control. No other bands were observed in samples taken from transgenic plants and no crossreacting bands were observed in tissue samples obtained from nontransformed *Arabidopsis* plants, thus, providing direct proof of the presence of the Lux F protein in transgenic *Arabidopsis* plants. A very heavy staining band of approximately 34 kDa was detected from the *E. coli* positive control sample. This protein has the same molecular weight has the β-subunit of the Lux AB protein and is evidence of the independent production of the β-subunit which has already been reported to occur in *E. coli* cells containing this vector (Escher *et al.*, 1989).

III.8 MONITORING LUCIFERASE ACTIVITY INDUCED BY PHYTOPATHOGENIC BACTERIA, *IN PLANTA*, USING A LOW-LIGHT IMAGER

Healthy, 1-2 week-old, transgenic *Arabidopsis* plantlets were grown and maintained on sterile MS 1.25% medium supplemented with kanamycin. These plants were imaged simply by removing the plastic Petri-plate lid, replacing it with a Pyrex™ Petri-plate lid bearing four to five 3 µL drops of *n*-decanal (99% pure) placed evenly on the inside. The length of the imaging with the low-light camera was 10-15 mins with the camera set at maximum sensitivity. Transgenic plantlets #2, #3, #5 and #6 did exhibit elevated levels of luminescence when compared to levels found in nontransformed control *Arabidopsis* plantlets. The younger plant tissue, adjacent to and within the apical meristem, often showed the highest levels of luminescence. After the timed photon collection, the Pyrex™ Petri-plate cover was replaced with the original plastic cover and the plants in the Petri dish were returned to the growth room. The decanal vapours which have already been noted to diffuse into plant tissue quite readily (Koncz *et al.*, 1990), penetrated into the young plant tissue and in fact had a perceptible toxic effect on the plantlets. After 24 hrs, when photon collection was to be carried out again, it was discovered that in a large proportion of the plantlets had died. The leaves farthest from the central rosette appeared most sensitive and had yellowed, while the only viable areas appeared to be the central tissue surrounding the apical meristem. Even 1-2 hrs after the first round of imaging and the initial exposure to *n*-decanal vapours, tissue damage to the plant was noticed as leaf wilting. The disadvantage of imaging plants grown and maintained in Petri plates is that *n*-decanal vapour, that penetrates into the plant tissue, cannot later diffuse out of the closed Petri dish, thus, continual exposure to residual *n*-decanal vapour within the Petri dish results in continuing plant tissue damage. The obstacle of decanal toxicity versus luminescent signal was also briefly discussed by Millar *et al.* (1992) with tobacco seedlings.

Successive experiments were carried out by allowing plantlets to mature for 4-5 weeks on sterile MS 1.25% supplemented with kanamycin. The photons from 4-5 week-old mature plants were collected and the mature plants showed a less sensitivity to a 10% *n*-decanal solution than seedlings, however, very low levels of luminescence were observed in mature plants of the different transformed lines, even when using an undiluted decanal solution. Based on these data, *PAL1* promoter expression gradually declines in leaf tissue in mature plants; this property was also noted by Ohl *et al.* (1990).

Analysis of *PAL1* promoter-*lux F* induction by bacterial infection with avirulent and virulent *P. syringae* strains was initiated on mature 4-5 week-old plants grown *in vitro*, however, under these conditions bacterial disease symptoms of leaf tissue were often atypical and not reproducible. This was attributed to the environmental conditions *in vitro*. Consequently, further analysis of bacterial induced *PAL1-lux F* expression in *Arabidopsis* plants was not performed in this manner.

Shortly thereafter, whole leaves were removed from 4-5 week-old *Arabidopsis* plants and these detached leaves were infiltrated on the middle edge of one side as described previously (Materials and Methods, II.13.1). These experiments were carried out with leaves from transgenic plant lines #2 and #5 and increases in luminescence in infiltrated leaves were observed and appeared to occur in two phases. An initial scarcely detectable increase in luminescence was observed in leaves 5 days after bacterial infiltration. At 12 days post-inoculation, prior to complete bacterial colonization of the leaf, substantial increases in luminescence were easily detectable (Giacomin and Szalay, 1993). Leaves infiltrated with *Pst* demonstrated 3.4-fold increase in luminescence, while *Psm*-infiltrated leaves demonstrated only a 2-fold increase compared to the uninfiltrated control leaves. These experiments demonstrated that mature *Arabidopsis* leaves are significantly more tolerant to *n*-decanal vapours than plantlets and that detached leaves can be monitored *in vivo*, for a period of several weeks, for changes in *PAL1-lux F* expression with no detectable leaf tissue damage resulting from *n*-decanal vapour.

However, the bacterial infiltration of detached leaves has the main disadvantage of atypical results with respect to bacterial pathogenesis symptoms, although *PAL1-lux F* expression still displayed familiar selective induction patterns. Therefore, to maintain the characteristic bacterial disease symptoms and the active physiological and biochemical function of the *Arabidopsis* leaf tissues, it was concluded that the infiltrated leaves should not be detached from the intact plant while the infection process is occurring. If possible, leaves should only be removed from the plant prior to assaying as was performed when carrying out the *in vitro* luciferase assay. Although increases in luminescence can be monitored *in vivo*, further experiments to examine bacteria induced *PAL1-lux F* expression were carried out exclusively in whole plants grown in soil under ideal greenhouse conditions.

Fully expanded, 4-5 week-old, greenhouse-grown plants (see Materials and Methods, II.13) chosen just prior to flowering, were examined for bacterial induction of the *PAL1* promoter-luciferase fusion gene, *in planta*. One leaf per plant was infiltrated with *Pst* and *Psm* as described previously. The photons emanating from the entire surface of the plant were captured by the low-light imager. Initially these experiments were carried out using transgenic plant line #2. In general, the levels of luminescence were found to be insubstantial. Experiments *in planta* were temporarily discontinued due to these negligible levels of luminescence. Transformed plant line #5 exhibited specific luciferase activities, *in vitro*, comparable to those observed in plants from transgenic line #2; both possess a single T-DNA insert. It was expected that any increases in *PAL1-lux F* gene expression in #5 plants, due to microbial infection, would also be, for the most part, undetectable. Transgenic line #6 displayed levels of luciferase activity that were much lower than #2 and #5 and would, therefore, also not be detectable using the low-light imager, *in planta*. Transgenic line #3, which possesses three T-DNA inserts and exhibited *in vitro* luciferase activities of approximately three times as high as those observed in #2 and #5, was selected for further experimentation using the low-light imager. There was a reasonable chance that these higher levels of luciferase activities would be detectable *in planta*, using the low-light imaging camera.

Transgenic #3 plants were infiltrated with avirulent and virulent *P. syringae* strains and were assayed using the low-light imager as described above for #2. The results demonstrated that there were indeed increases in the luminescence from infiltrated leaves and that these increases were detectable by a low-light imager, *in planta*. In this experiment it was noticed that the dry, brown, papery, hypersensitive lesions, caused by *Pst*, 24 hrs post-infiltration, were typically surrounded by high levels of luminescence; those areas which were lightly-yellowing or lightly-browning, in the early stages of HR necrosis, showed the highest levels of induced luminescence (see Figs. 16 and 17, the luciferase analysis at the 48 hrs and 72 hrs post-inoculation sampling points show similar levels of bioluminescence to the 24 hrs time point and are not shown). No induced luminescence was observed in the already dead, brown, necrotic tissue at the centre of a lesion, or in uninfiltrated leaves indicating that the *PAL1* promoter is not induced in the centre of the hypersensitive lesion, or systemically in any uninoculated tissue of the same plant. Inoculation with the virulent strain, *Psm*, also increased levels of bioluminescence adjacent to the lesions, but to a notably lesser degree. Characteristically, the virulent bacteria caused necrotic lesions surrounded by a chlorotic halo. Chlorotic haloes, typically spread out to engulf the whole leaf resulting in leaf-death within 5-7 days. It is interesting to note that those areas which had the highest induced levels of luciferase activity in leaves infiltrated with the avirulent bacteria, *Pst*, were adjacent to necrosed tissue and were generally green healthy tissues, or were tissues which were in the early stages of the HR and were lightly yellowing. In contrast, the same areas of leaves

Figure 16: Monitoring in "real-time," *PAL1-lux F* expression *in planta*, in *Arabidopsis* leaves infiltrated with *P. syringae* pv. *tomato*. *A. thaliana* (transgenic plants #3) containing 3 copies of the *PAL1-lux F* transcriptional fusion were used. A leaf from a 4-5 week-old plant was infiltrated with *Pst* and the whole plant was "imaged" for ten minutes, 24 hrs after leaf infiltration. Expression of the transgene is observed on the perimeters of the necrotic lesion; ***a***- shows the beginnings of the formation of a local brown necrosis observed in the leaf at the infiltration site, 24 hrs after infiltration, this photograph was taken under normal light; ***b***- shows a photon image collected for 10 mins of the whole plant in *a* (the colour calibration bar indicates number of photons increasing from bottom to top); ***c***- a magnified view of *b* of the area of greatest bioluminescence; ***d***- a video image of the same plant superimposed upon the photon image.

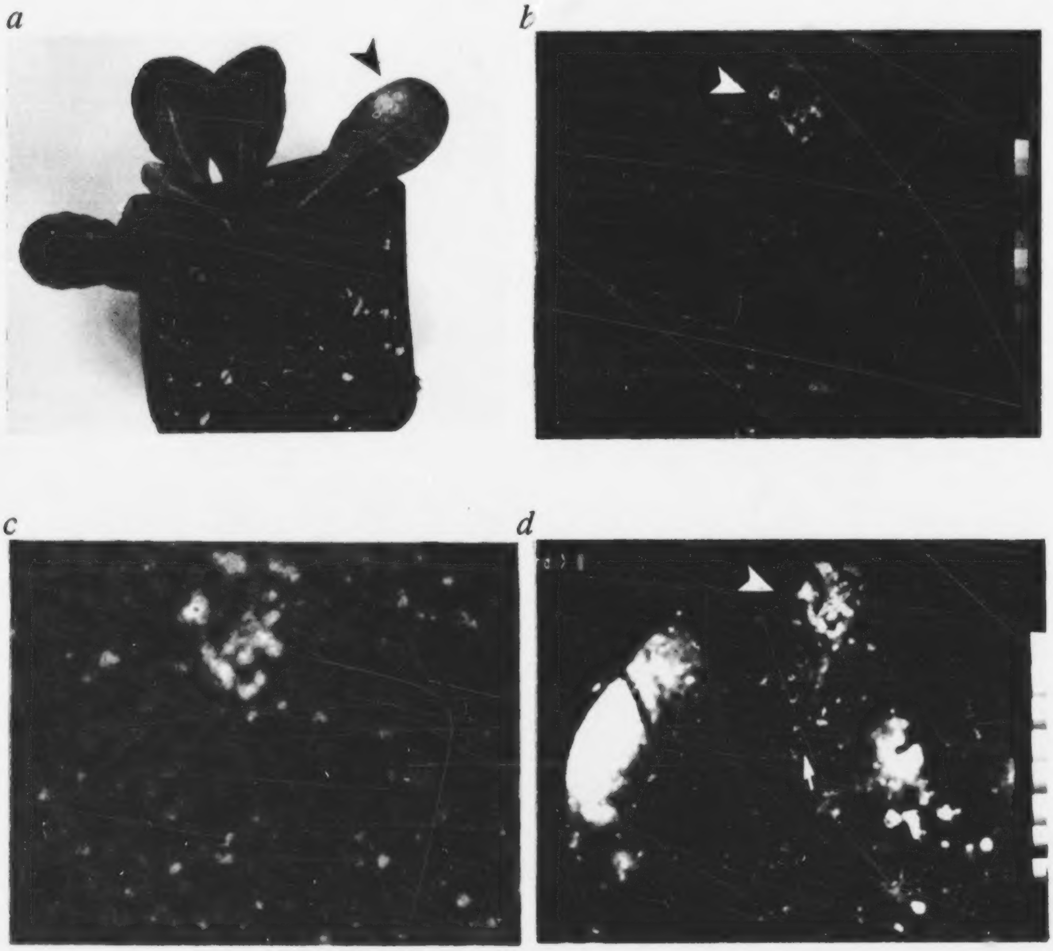
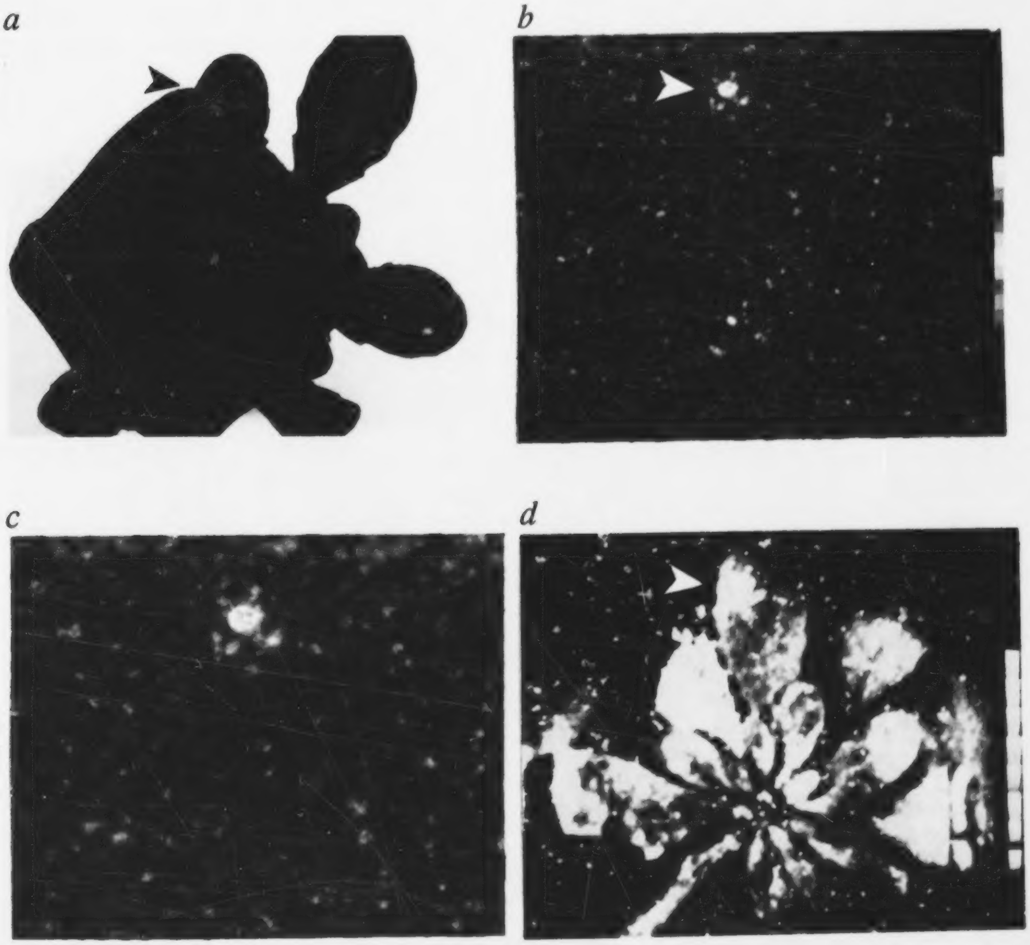


Figure 17: Monitoring in "real-time," *PAL1-lux F* expression *in planta*, in *Arabidopsis* leaves infiltrated with *P. syringae* pv. *maculicola*. *A. thaliana* (transgenic plants #3) containing 3 copies of the *PAL1-lux F* transcriptional fusion were used. A leaf from a 4-5 week-old plant was infiltrated with *Psm* and the whole plant was "imaged" for ten minutes, 24 hrs after leaf infiltration. Expression of the transgene is observed on the perimeters of the necrotic lesion; ***a***- shows the beginnings of the formation of a local brown necrosis observed in the leaf at the infiltration site 24 hrs after infiltration, the photograph was taken under normal light; ***b***- shows a photon image collected for 10 mins of the whole plant in *a*, (the colour calibration bar indicates number of photons increasing from bottom to top); ***c***- a magnified view of *b* of the area of greatest bioluminescence; ***d***- a video image of the same plant superimposed upon the photon image.



infiltrated with the virulent strain showed lower levels of luciferase activities and eventually became chlorotic and died 3 days later. These increased luciferase activities were present up to 5-7 days after infiltration with *Pst* and the lesion grew only slightly. At 5-7 days post-infiltration, the leaves infiltrated with the *Psm* were soggy, drooping and entirely chlorotic, hence, photon collection was not performed since the tissue was for the most-part dead. In some instances, low-light imaging analysis did reveal high levels of luciferase activity at the base of the leaves infiltrated with *Psm*, in the small portions of remaining living tissue 3-5 days after infiltration.

The measurements of luminescence collected *in planta* and calculated over the entire area of the leaves 2 days after infiltration with *Pst*, displayed a higher level of bioluminescence of 20335 photon counts /leaf (photon count for 10 mins, n=2), while the average luminescence detected in adjacent uninfiltrated leaves was only 9128 photon counts/leaf (photon count for 10 mins, n=2). The maximum bioluminescence in leaves infiltrated with the *Pst* was calculated to be 2.23 times higher than background uninfiltrated leaves 48 hrs after infiltration. Leaves infiltrated with the virulent bacteria, *Psm*, showed a maximum induced luminescence 24 hrs after infiltration of 14878 photon counts/leaf (photon count for 10 mins, n=2), 1.63 times the background. The relative increases of the induced luminescence monitored *in planta*, do not differ significantly from the values recorded by way of luminometry; although the increases in luminescence in *Psm*-infiltrated leaves appeared to be slightly higher, while *Pst*-infiltrated leaves appeared to generate slightly lower luminescence than the increases noted by way of the *in vitro* luciferase assay. This apparent discrepancy is not significant since a small sample size in conjunction with irregular fluctuations in the luciferase activity of a specific sample can produce that result.

As stated previously, the induced bioluminescence was confined to local areas surrounding necrotic lesions. Since the quantities of bioluminescence issuing from the whole leaf surface were calculated, understandably the local increases in luminescence are far greater than the values stated. The luminescence emanating from the small local regions was found to be 10-20-times greater (depending on the area examined), than the average induced luminescence calculated over the whole leaf surface area (see Figs. 16 and 17). Note that the colour calibration bar in Figures 16 and 17 (*b*), gives a linear scale from 1-15, thus, the areas of red are radiating 12-fold more photons than areas of dark blue; the superimpose pictures in Figures 16 and 17 (*d*) allow localization of areas of high photon-emission in *b* onto the plant, but do not themselves provide accurate quantitative information. Logically, the smaller the area that was chosen and the more confined to the "hot-spots", the higher the value of luminescence averaged per that specific area. Maximum values of approximately 9 000 photons were recorded surrounding lesions; these confined areas were equivalent to approximately 5% of the total leaf area.

During the 3-5 consecutive daily assaying period no harmful effects on plant tissue, as a result of decanal exposure, were apparent. The 2 minute pre-incubation period in the dark low-light imaging chamber, which was required for the extinction of chlorophyll luminescence, was found to be entirely adequate for decanal vapour penetration into the *Arabidopsis* plant tissue. No lengthy pre-incubation period, which is required for penetration of luciferin substrate for the firefly luciferase assay (Millar *et al.*, 1992), was needed for decanal vapour penetration in this study. After imaging, plants were removed from the decanal vapour-filled jar and were promptly returned to the greenhouse to allow the decanal vapour absorbed into the plant tissue to evaporate. The toxic effects of decanal vapour exposure, as were observed when iamging plants grown and maintained in Petri plates, were precluded with the greenhouse-grown plants. Mature *Arabidopsis* plants can be exposed to high concentrations of *n*-decanal vapours for 10-12 mins on a daily consecutive basis for 4-5 days. This procedure allows for minimal handling of plants which remained in the pots in which they had grown and were not subjected to any changes in growth conditions.

Interestingly, an unrelated phenomenon was observed: several uninfiltrated leaves from apparently healthy #3 plants developed a light red colour over much of the leaf surface while growing in the greenhouse. This pigment was visible to the naked eye and was interpreted as evidence of the production of a possibly "stress-related" anthocyanin-derived pigment. These leaves were also examined using the low-light imager and displayed extremely high levels of luminescence of approximately 15-20-fold higher than the levels found in normal healthy leaves. The increased luminescence was found over most of the leaf surface area, but was especially situated in the red-coloured areas; thus, a correlation was found between the production of a red pigment (probably phenylpropanoid in nature) and *PAL1* promoter activation.

Also of interest was the extremely high levels of luminescence detected at the base of the inflorescence. At the initial stages of inflorescence growth induced levels of bioluminescence were observed; luminescence levels increased until the inflorescence stalk had reached its maximal elongation. As stated previously, the inflorescence base possesses a woody-type consistency and is an extremely rigid plant tissue which is not characteristic of the other vegetative parts of the *Arabidopsis* plant. Based on the properties of this tissue it is probable that a high degree of lignification occurs at the base of the inflorescence as a mechanism for strengthening the plant cell walls to provide additional support to carry the heavy multi-branched flowering stem. At the base of the inflorescence, and up to 1-2 cm above the rosette, the levels of luminescence in transgenic *Arabidopsis* plants measured *in planta*, reached a maximum of approximately 10-20-fold higher than background. Similar levels of induction were observed in leaf tissue caused by bacterial infiltration, however, in this case, luminescence was confined to local regions rather than being widespread as was observed throughout the stem tissue. The differences in magnitude of the *in vitro* measurements of luciferase enzyme activity, discussed previously between stem-tissue and the leaves infiltrated with the avirulent bacteria (III.7), are thus due to the widespread tissue expression in the first case versus the local tissue expression of *PAL1-lux F* expression in the second. It is important to note that a 1-2 cm stem section with high *PAL1-lux F* expression throughout, measured *in vitro* using an enzymatic luciferase assay, generates photons from luciferase protein synthesized throughout the tissue, while photon counting *in planta* generates data from the surface area of the stem immediately beneath the camera lens. Any photons generated by luciferase protein below the upper surface of the stem section would definitely be subject to quenching by the tissue above it (the stem grows vertically). Hence, luciferase expression can be subject to quenching, especially when thick sections (>1 cm) of tissue are being examined.

Similar observations of PAL gene promoter activation have been noted previously. Bevan *et al.* (1989) observed high levels of β-glucuronidase activity in the stem sections of transgenic tobacco plants containing the bean *PAL2* promoter fused to the β-glucuronidase gene. In addition, staining for lignin showed that these tissues contained lignin deposits. It was postulated that PAL activity in these leaves could provide intermediates in lignin biosynthesis. Lignification requires phenolic precursors generated by the phenylpropanoid pathway, especially phenylalanine ammonia lyase. This requirement is satisfied by the transcriptional activation of the PAL gene mediated by its promoter and is reflected in the high levels of *PAL1* promoter activation.

The results of the measurements of luminescence *in planta*, which showed increased levels of luminescence due to microbial infiltration, paralleled the results of the quantitative luciferase assays performed *in vitro*, using whole leaves. These results revealed specific increases in *PAL1-lux F* expression caused by inoculation of an avirulent microbe, but not a virulent microbe. Therefore, the data obtained from the low-light imager of plant leaves *in planta*, do reflect the specific levels of luciferase enzyme present in leaves. Also, the *in planta* monitoring system can provide additional data of stress-induced regulation (in the case of red pigment production) and novel information on the developmental/tissue-specific regulation (in the case of inflorescence growth) of the *PAL1* promoter in *Arabidopsis*.

IV. DISCUSSION

IV.1 FINDINGS

Quantitative measurements of the luciferase activities of leaves from transgenic *Arabidopsis* plants demonstrated several important features. The basal levels of luciferase activities of leaves from transgenic *Arabidopsis* plants, transformed with the *PAL1-lux F* construct, was found to be 10 times greater than the basal levels of luminescence from nontransformed control *Arabidopsis* plants. This finding, along with the fact that the Lux F protein was directly detected in stem tissue homogenates, demonstrates that the the Lux F protein is present in transgenic plants and is functionally active. On average, a rapid 3-4-fold increase in luciferase activities was detected in leaves inoculated with *P. syringae* pv. *tomato* within 24 hrs after bacterial infiltration. The consistent increases in the luciferase activities of leaves, from four separate transgenic lines, infiltrated with *P. syringae* pv. *tomato*, proved that there is in fact a specific mechanism for the induction of the *PAL1* promoter, which is triggered shortly after infiltration with these bacteria, resulting in increased reporter gene activities. The virulent bacteria, *P. syringae* pv. *maculicola*, elicited only minor increases in luciferase activities in transgenic *Arabidopsis* plants. These minor increases decreased to basal levels 48 hrs after bacterial infiltration. The leaves "mock-inoculated" with $MgCl_2$ showed only slight transient increases in luciferase activities; these decreased rapidly to basal levels 48 hrs after infiltration. Thus, the transformed *PAL1-lux F* transcriptional fusion construct is functioning in plants in a specific regulated fashion. The data gathered in these experiments closely parallels the work of Dong *et al.* (1991), who report an 8-fold increase in PAL mRNA, induced in *Arabidopsis* leaves (ecotype Colombia) infiltrated with *P. syringae* pv. *tomato*, over the PAL mRNA levels in leaves infiltrated with *P. syringae* pv. *maculicola*. Although, the mRNA induction levels are not directly comparable to absolute increases in the synthesis of protein, the kinetics and specificity of induction of luciferase activity in this study and PAL mRNA induction shown previously coincide. The presented data of *Arabidopsis PAL1-lux F* activation corresponds to increases in PAL activity reported in other plant systems; for example, 3.33-fold increases in PAL activity in resistant tomato petioles were observed over the PAL activity determined in susceptible tomato petioles, 24 hrs after inoculation with *Verticillium alboatrum* (Lee *et al.*, 1992); 3.25-fold increases were observed in PAL activity in lignifying wheat leaves 16 hrs after wounding and application of fungal-derived elicitor (Mitchell *et al.*, 1994); 3.4-4.1-fold increases in PAL activity was described in resistant potato tubers, over the PAL enzyme levels of non-resistant potato tubers, 1-3 days after inoculation with *P. infestans* (Henderson and Friend, 1979). In two similar cases, excised bean pods and soybean seedlings exhibited approximately 6.9-fold and 7.28-fold increases in PAL activity, respectively, after inoculation with phytopathogenic fungi (Hess *et al.*, 1971; Biehn *et al.*, 1968).

Arabidopsis contains 4 PAL genes and it remains unknown whether the other PAL genes are activated in a similar manner to the *PAL1* gene. There may exist a selective induction mechanism for the activation of specific PAL genes, in response to microbes, due to subtle differences in promoter structure, as was shown previously with two *PAL* promoters from bean (Shufflebottom *et al.*, 1993). Future research of the genetic regulation of phenylpropanoid metabolism in *Arabidopsis* should centre on the cloning of the remaining 3 PAL genes from *Arabidopsis* and the determination of their respective promoter induction patterns.

The data collected by way of luminometry does provide quantitative analytical values for the induction of the luciferase reporter enzyme in whole leaves. However, the specific area of *PAL1* promoter induction in the leaf, with respect to the bacterial infection site, cannot be assessed in this way, nor can a single leaf be monitored repeatedly since the enzymatic measurements require the destruction of the sample which allows only a single measurement of a leaf. Consequently, to monitor and determine the spatial and the temporal regulation of the *PAL1* promoter within a specific leaf, low-light imaging analysis *in planta* was performed.

The low-light imaging analysis showed that *PAL1* promoter is activated in discrete areas encircling the infiltration site. The increased luciferase activities, which were recorded by means of luminometry and could be inferred as occurring within the whole leaf, were in fact found not to occur over the entire leaf surface, but were confined to local areas adjacent to the microbial infection. Sharp borders were observed between the infection site centre, where hypersensitive cell-death had occurred in response to bacterial infiltration, the surrounding areas of local gene activation and the remainder of the healthy leaf tissue which did not show any detectable response. Elevated levels of luminescence, 10-15-fold higher than surrounding leaf tissue, were observed in local areas indicating a pronounced, but local *PAL1* promoter induction .

Similar observations of local activation of plant defense genes were recorded in potato and parsley leaves infected with the fungi *P. infestans* and *P. megasperma* f. sp. *glycinea* (Cupyers *et al.*, 1988; Schmelzer *et al.*, 1989). Using *in-situ* hybridization induced PAL mRNA was observed to reach maximum levels between 3-6 hrs and between 6-8 hrs post fungal-inoculation, respectively; and was distinctly localized around the necrotic spot which itself was free from any hybridizing signal. Cupyers *et al.* (1988) observed that PAL activation in the susceptible potato variety was delayed more diffuse and nontransient; and Schmelzer *et al.* (1989) concluded that the furanocoumarin phytoalexins are not among the earliest defense responses, instead, the accumulation of esterified phenolics in the cell walls of responding plant cells may occur earlier. Phenolic polymers and esterified phenolic acids have been shown to accumulate extremely rapidly in plant cell walls immediately proximal to elicitor treatment and to reach maximal levels when phytoalexin production was only just beginning (Graham and Graham, 1991).

IV.2 SIGNIFICANCE

The results of these experiments employing a *PAL1 - lux F* transcriptional fusion in *Arabidopsis* provides additional information of the spatial properties of defense gene activation in plants in response to microbial infection. The border in transgenic *Arabidopsis* leaves between the tissue highly expressing luciferase, directly around the lesion towards the central necrotic spot, and outwards towards the unaffected tissue expressing normal levels of luciferase, were distinct. It is highly probable that the activation of the *PAL1* gene surrounding the infiltration site is a mechanism for containing the bacteria at the infection site. One can postulate that in this ring of tissue surrounding an infection site, various defense genes, including *PAL1*, are activated possibly resulting in the production of plant cell wall-bound phenolics, phytoalexins and increased deposition of lignin. PAL activity is known to be associated with cells that are undergoing lignification of the secondary thickenings of the cell walls, as lignin is a polymer composed of phenylpropanoid monomers (Bevan *et al.*, 1989). Also of related significance a phytoalexin called camalexin was recently identified and was found to be synthesized *de novo*, in *Arabidopsis* leaves, upon bacterial inoculation (Tsuji *et al.*, 1991). Camalexin was found to accumulate rapidly in infected leaves, between 12-24 hrs after inoculation and to reach maximal levels 24-48 hrs post-inoculation. Thus, there is a correlation between the transcriptional activation of an *Arabidopsis* defense gene (i.e., *PAL1* promoter activation), which occurs shortly after exposure to microbes and the induction of other *Arabidopsis* defence responses such as camalexin synthesis and increased *ELI3* mRNA (Tsuji *et al.*, 1991; Kiedrowski *et al.*, 1992). Based on the results presented here of localized *PAL1* induction, I submit that the active defense mechanisms described in previous reports, of *de novo* phytoalexin synthesis and of increased transcription of PAL genes and *ELI3*, which have been examined on a per leaf basis in *Arabidopsis* using destructive assaying methods, are also actually occurring in a localized band of cells surrounding the infection site.

Another recent example demonstrates that an *Arabidopsis* acid chitinase gene is also activated in a sharp band of healthy cells, surrounding fungal lesions in transgenic *Arabidopsis* and tomato plants containing an *Arabidopsis* acid chitinase promoter transcriptionally fused to a β-glucuronidase (GUS) reporter gene (Samac and Shah, 1991). GUS staining of *Arabidopsis* leaves was carried at various time intervals after inoculation with *Rhizoctonia solani*. Three days after inoculation, when strong lesion development was plainly visible, intense GUS staining was detected in the mesophyll layer bordering the diseased tissue; GUS staining in the remaining healthy tissue at some distance from the lesion remained unchanged. In transgenic tomato plants, similar overall patterns of fungus-induced chitinase promoter activation were noted as in bacterial-induced *PAL1-lux F* expression in transgenic *Arabidopsis* plants. In tomato transformed with an acid chitinase promoter-GUS gene fusion, GUS activity was not detected around the large coalescing lesions 10 days after inoculation, suggesting that this promoter activity is transient and linked to early necrotic lesion development. This finding differs from the findings in this study of *PAL1* promoter activation in *Arabidopsis*. In several cases strong luciferase activity was observed in the last remnants of healthy tissue surrounding large coalescing lesions in leaves inoculated with *Psm*; highly elevated luciferase activities were also observed in leaves inoculated with *Pst* 5-7 days after leaf infiltration surrounding the slowly expanding, dry, papery, lesion. The *PAL1* promoter appears to be induced in the later stages of both the incompatible and the compatible reaction, when extensive tissue damage has occurred, although at this stage *PAL1* promoter induction does not appear to have any consequences in the final outcome of leaf infection.

The general conclusions forwarded by Schmelzer *et al.* (1989) of defense gene activation in parsley and observations of fungal activation of a chitinase promoter in *Arabidopsis* (Samac and Shah, 1991) appear to apply to bacterial infection in *Arabidopsis* plants shown in this thesis and are probably characteristic of defense gene activation common throughout the plant kingdom. First, the hypersensitive cell death at the actual site of the penetration by the microbe is too rapid to involve the activation to a measurable extent of the PAL gene or any other identified defense gene, therefore, the hypersensitive response is more likely the result of a direct interaction of the invading microbe with the plant cells and hence is prominently localized. Secondly, the sharp borders between cells strongly activated to produce luciferase and cells which have not activated the *PAL1* promoter, indicates a threshold phenomenon in the signalling mechanism. It has become more and more apparent that the events that determine host-resistance or susceptibility, often occur in very limited population of cells at, or just in advance of the infection front, in the very narrow zone defining the area of immediate contact between host and pathogen (Graham and Graham, 1991). Interactions occurring after an infection is established and is progressing through a tissue may be largely irrelevant to the outcome of the infection. Graham and Graham (1991) state: "it is no longer sufficient to measure responses by gross tissue analysis, even in those cases where they represent discrete lesions, since these lesions may represent a composite of many cellular and molecular events". Two primary approaches of analyzing the cellular aspects and locations of local gene induction currently in use are: i) analysis of specific mRNAs by *in situ* hybridization, and ii) *in planta* analysis of promoter-reporter gene fusions in transgenic plants. A limitation of *in situ* hybridization is that it does not provide an analysis of the actual accumulations of the defensive proteins or metabolic products that follow gene activation. Reporter gene analysis may provide information which is indicative of the levels of accumulation of defense proteins, although, the accumulation of secondary enzyme products (i.e., phenolic compounds) cannot be analyzed using this technique. Also, reporter gene analysis, *in vivo*, can provide the experimenter with a real-time analysis of experimental, thus, in some instances, the rapidity and ease of reporter gene analysis can make it more desirable over *in situ* hybridization where tissue samples are destroyed for analysis.

The basis of the specificity of cultivar-specific resistance at the genetic or molecular level still remains unclear. Current theory suggests that an active recognition process occurs at the site of contact between plant cells and microbes. The induction of transcription of defence genes, such as PAL, has become the focus of investigation in recent years. The general consensus of much previous work demonstrates that the PAL genes, as well as other plant defense genes, are activated by: i) wounding, ii) exposure to elicitors (microbial derived or abiotic), iii) exposure to UV-light, iv) exposure to microbial pathogens, or v) by any combination of the forementioned treatments (Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Lamb *et al.*, 1989; Schmelzer *et al.*, 1989; Dixon and Harrison, 1990; Keen, 1992). Significantly, differences in the nature of PAL gene activation in response to avirulent and virulent microbial pathogens have also been described. Several reports in the past have shown that defense genes, including PAL (and chalcone synthase), are preferentially induced earlier in response to inoculation with avirulent strains over virulent strains (Bell *et al.*, 1986; Esnault *et al.*, 1987; Lawton and Lamb, 1987; Jahnen and Hahlbrock, 1988; Habereder *et al.*, 1989; Voisey and

Slusarenko, 1989; Benhamou *et al.*, 1990a, 1990b). More recently it has been demonstrated, specifically in *Arabidopsis*, that the time course of induction of defense genes (e.g., PAL and *ELI3*) are similar, however, inoculation with avirulent strains of pathogen induce gene expression to a far greater extent (Dong *et al.*, 1991; Kiedrowski *et al.*, 1992). In a contrasting manner, other reports suggest that virulent microbial pathogens are able to actively suppress PAL gene activation (Yamada *et al.*, 1989; Lee *et al.*, 1992; Jakobek *et al.*, 1993). Thus, the precise nature of host-specific PAL gene activation in plants, in response to distinct races of microbial pathogens, still remains uncertain and is under investigation. The results of this work support the work of Dong *et al.* (1991) and Kiedrowski *et al.* (1992) and demonstrate a preferential activation of a plant defence gene by avirulent bacteria, in comparison to virulent bacteria, although the kinetics are identical. There now exists a facile system for testing these hypotheses and any other hypothesis of plant-microbe recognition in *Arabidopsis* plants by using a variety of different microbes, including non-phytopathogenic organisms, to test theories and models of plant-resistance to microbes. *Arabidopsis* has become established as a model plant system for use in studying a variety of molecular phenomena in plants, in particular *Arabidopsis-Pseudomonas* interactions have recently become established as the model plant-pathogen system (Dong *et al.*, 1991; Davis, 1992). This work is another contribution to the ever expanding field of plant-pathogen research in *Arabidopsis*.

IV.3 POSSIBLE IMPROVEMENTS

The first attempts at collecting the photons from plants *in planta*, by placing plants from transgenic line #2 in a closed glass jar which contained *n*-decanal and collecting photons using a low-light imager was not successful since the levels of luminescence coming from the infiltrated leaves were not of sufficient intensity to be imaged efficiently. Based on this experiment and comparisons to the *in vitro* luciferase assays, it was concluded that the levels of induced luminescence coming from transgenic line #3 (a transgenic line which contained three T-DNA insertions and therefore three copies of the *PAL1-lux F* reporter gene construct), might be intense enough to be detectable *in planta*, using the low-light imager. In fact, the luminescence coming from infiltrated leaves of #3 transgenic plants was detected *in planta*. The preceding discussion of the properties of the spatial activation of the *PAL1* promoter in *Arabidopsis* leaves infiltrated with microbes is based solely on observations gathered *in planta*, from transgenic line #3, since the other three transgenic lines were not generating intense enough luminescence to be analyzed *in planta.* Reporter genes in general are fraught with their own specific obstacles and each reporter gene system has its advantages and its disadvantages. In plant tissue the spatial patterns of promoter activation can be deduced by enzymatic assays. However, the results of GUS histochemical analysis, or imaging of bioluminescence (bacterial or firefly luciferases) are often difficult to compare directly (Nilsson *et al.*, 1990). The arrangement deduced from enzymatic measurements varies with the basis used to express the activity (Jefferson *et al.*, 1987; Schneider *et al.*, 1990). The patterns revealed by histochemical analysis can be affected by such factors as the method of fixation, localized occurrence of degradative enzymes, *in situ* reaction conditions and diffusion of reaction products (Nilsson *et al.*, 1990). In the case of light emission, the pattern detected by imaging apparatus can be affected by reflection, quenching and uneven substrate distribution. Thus, no reporter gene system is "flawless" and a specific reporter gene system should be chosen depending upon the immediate limitations or confines of current experiments. Ideally, a promoter under study should be fused to different reporter genes and the expression of all the reporter genes should be monitored in parallel in the same plant (Nilsson *et al.*, 1992). In a preliminary study of the *Arabidopsis PAL1* promoter, Ohl *et al.* (1990) constructed a transcriptional fusion between the *PAL1* promoter and the GUS reporter gene. This construct was reintroduced into *Arabidopsis* plants and transgenic plants were analyzed for increased GUS activity in response to various stresses, including wounding, $HgCl_2$-stress and UV-light exposure. Though the authors did detect increased transcripts of GUS mRNA after stress treatment, no increases in specific GUS activities were detected. Ohl *et al.* (1990) concluded that any transient increase in GUS activity, due to environmental stress, is probably not detectable against the high background of elevated GUS activity, which had accumulated during the plant's growth from seedling to full-grown plant. Therefore, the use of the GUS reporter gene system, which produces a relatively stable GUS protein, may not be a suitable reporter gene for studying transient increases in gene expression of the *PAL1* promoter in mature *Arabidopsis* plants, since the GUS protein can accumulate over prolonged periods during plant growth. Hence the main subject of this thesis: the use of the *lux F* reporter gene to monitor transient increases of *PAL1* promoter induction which could potentially provide a truly *in planta*, or non-destructive reporter gene system. Conventional transcriptional analysis necessitates the use of mRNA markers, or of destructive reporter gene such as chloramphenicol acetyl transferase, both of which require radioactive tracers and lengthy time-consuming procedures. More importantly, these procedures all involve the destruction of the experimental material. Molecular genetic techniques could be greatly accelerated if the transcriptional analysis of promoters could be performed by using a simple noninvasive assay for promoter activity. Consequently, the development and use of a truly non-destructive reporter gene assay system is of considerable interest and is the focus of continuing research. Luciferases (both bacterial and firefly) are the most versatile class of noninvasive reporter gene currently available; most efforts creating an *in vivo* reporter gene system have centred on their use (Legocki *et al.*, 1984; Langridge *et al.*, 1989; Koncz *et al.*, 1990; Millar *et al.*, 1992; Nilsson *et al.*, 1992). The truly non-destructive reporter gene system has been found to be inherently a much more difficult system to develop and to employ than destructive assays, especially in multicellular eukaryotic systems, for the very reason that the tissue under examination cannot be destroyed for analysis. Up to date there have been two reports of the use of luciferases to monitor plant promoter regulation *in vivo*. In one report Millar *et al.* (1992) used the firefly luciferase reporter gene to monitor the circadian regulation of the *Arabidopsis* cab2 promoter in transgenic tobacco, nevertheless, this investigation utilized tobacco seedings not fully grown plants and the authors comment that the penetration of the luciferin substrate in older plants, which possess thicker epidermal tissue-layers and surface-wax coatings, may pose a problem; thus, their system may be limited to plant seedlings. In another report Nilsson *et al.* (1992) use the bacterial luciferase (*lux F*) to monitor the expression of the 35S cauliflower mosaic virus promoter in the leaves of young poplar trees, albeit, the 35S cauliflower mosaic virus promoter is often designated a constitutive promoter and is highly expressed in plant tissue in an almost deregulated fashion. Their experiment does not demonstrate the use of bacterial luciferase to monitor an endogenous regulated plant defence promoter. The experiment discussed in this thesis is the first to report the use of the bacterial luciferase to monitor the induction of a regulated plant-defence promoter, *in planta*, in *Arabidopsis*. Previous published reports using luciferases (bacterial and firefly), besides the ones already indicated, were not really non-destructive since the tissue to be assayed was removed from the plant, to facilitate either the monitoring or the feeding of the substrate (Langridge *et al.*, 1990; Schneider *et al.*, 1990). Initially, attempts of using detached *Arabidopsis* leaves for monitoring *PAL1* promoter induction were carried out (Giacomin and Szalay, 1993). Atypical tissue disease symptoms and/or microbial growth in the detached *Arabidopsis* leaves were noted. For a plant-pathogen study using infiltrated leaves, it is more appropriate to examine plant responses *in planta*, thereby maintaining the operative physiological and biochemical function of the infected leaf tissue.

In transgenic plants which contain only one T-DNA insert, the levels of *PAL1* promoter induction resulting from microbial infection and the resultant synthesis of functional luciferase protein were found to be too insubstantial to be efficiently monitored *in planta*. However, for the transgenic plant line #3 containing three copies of the *PAL1-lux F* reporter gene construct, the specific luciferase activity was observed to be approximately triple the specific luciferase activities found in those plants with a single insert. This transgenic line did generate a strong enough photon signal in the inoculated leaves to be monitored *in planta* in mature *Arabidopsis* plants. The ability to monitor gene expression in a rapid manner, *in vivo*, has stimulated research to develop and improve reporter gene systems. Bacterial luciferases have already been identified as having a lower quanta yield than insect luciferases. The difference in the quanta yield of firefly luciferases and bacterial luciferases has been noted to be 0.1 versus 0.9 for the firefly system (Millar *et al.*, 1993; Ziegler and Baldwin, 1981). In other words, on an equivalent protein basis, firefly luciferases can generate approximately 10 times more photons than bacterial luciferases; therefore, for an equivalent amount of protein synthesized as a result of promoter activation, a photon signal of at least 10 times greater could potentially be generated. Although problems of access of the luciferin substrate into living tissue was identified as a potential obstacle of the firefly luciferase reporter enzyme (Subramani and Deluca, 1988; Koncz *et al.*, 1990). Also, it has been suggested that the stability of firefly luciferase in the absence of substrate may restrict its ability to be used as a reporter gene in higher plants (Quandt *et al.*, 1992). The advantage of easy substrate accessibility of the decanal substrate, required for bacterial luciferase, into living tissue, may override the disadvantage of lower quanta yield. Recently the isolation of luciferases from deep-sea marine organisms such as *Renilla* sp. (sea pansy) and *Aequoria* sp. (jellyfish), which appear to generate a quanta yield even higher than firefly luciferases, suggests the potential use of these proteins as reporter enzymes. The utility and advantages of employing bacterial luciferase as a non-destructive, or "live-time" means of quantifying gene expression during the course of cellular development, or in this case during the transcriptional activation of a plant defence gene, has lead researchers to seek to improve the bacterial luciferase reporter gene system. In fact researchers have endeavoured to increase the specific activity of the Lux F fusion protein, thereby increasing the quanta yield. In addition, the suggestion of increasing transgene copy-number in transformed plants has been recommended as a means of generating a higher photon signal (Okumura *et al.*, 1993). Also of potential importance are the results of Okumura *et al.* (1993), who have demonstrated that the incorporation of the Omega-3 translational enhancer sequence (functionally equivalent to the prokaryotic Shine-Dalgarno sequence) directly upstream of the *lux F* reporter gene in the transcribed, but nontranslated region of mRNA, results in 5-fold increases in the amount of protein synthesized from *lux F* mRNA molecules. This type of translational enhancement resulting in greatly increased yields of Luciferase protein, could prove extremely effective in increasing the photon-signal in specific systems and with reference to this study, perhaps allow microbial-induced *PAL1* promoter activation to be monitored *in planta*, even in those transgenic plants which contain only one T-DNA insert. Another suggestion that has been proposed to improve the *lux F* reporter gene system is the use of other chemical substrates. The possibility of using different aldehyde compounds, which may be suitable substrates for the luciferase enzyme, yet have a significantly reduced toxicity, may be of some future importance.

IV.4 ADDITIONAL FINDINGS

This study also demonstrates the relationship between the number of reporter gene integrations and the resultant reporter gene activity. Three T-DNA inserts in one transgenic line resulted in approximately 3-fold higher luciferase activities than transgenic plants with only one T-DNA insert, thus reporter gene activity appears to be closely related to the number of copies of transgene. In one transgenic line multiple T-DNA insertions were observed, but reporter gene activities in mature plants did not appear to be related to the number of transgenes and were generally noted to be equal to background. Based on Southern hybridization data it was determined that this transgenic line (transgene line #6) contained multiple insertions in a direct "head-to-tail" orientation. Therefore it appears the levels of reporter gene activity, in general, are affected by the number of reporter gene copies, nevertheless, the structure of T-DNA integration is also significant and can exert a "silencing effect" on transgene expression. Of significant interest was the apparent uncharacteristic decline in luciferase reporter gene activities in leaf tissue as plants from this transgenic line matured, indicating that the phenomenon of "gene-silencing" is a dynamic process and increases with plant age. It is sometimes observed that shortly after plant regeneration, a particular transformant may be observed to be producing high levels of reporter enzyme activities early on in the transformation process. However, when the plant is allowed to mature and reporter enzyme levels are again measured, no reporter enzyme activity is detected. Gene-silencing could possibly be the cause of the apparent loss in reporter gene expression which is sometimes observed during plant transformation procedures in specific individual transformants. Every plant transformant should be examined by way of Southern analysis to verify the number and structure of T-DNA insertions and to be aware of the potential for large decreases in gene expression, especially in those transformants which contain multiple juxtaposed T-DNA inserts. The *PAL1-lux F* construct used in this study could be employed to further examine the phenomenon of gene-silencing in plants.

IV.5 CONCLUDING REMARKS

The experiments presented in this thesis had a general objective of familiarization and preliminary training in the current techniques used in plant molecular biology. Specifically the objective of this project was to construct a novel *in vivo* system to monitor *PAL1* promoter induction in *Arabidopsis* plants in response to microbial infection. The PAL enzyme is the first and therefore, the key enzyme in the phenylpropanoid pathway. The transcriptional regulation of the PAL genes, in the case of microbial infection, is of extreme importance in determining the signal transduction pathway, elucidating the plant resistance mechanism and identifying novel bacterial elicitors. It is now clear that the *lux F* reporter gene can function in *Arabidopsis* plants under the transcriptional control of the *PAL1* promoter and that increases in luciferase reporter gene activity due to *PAL1* promoter activation can be monitored *in planta.*

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